SOME ASPECTS OF CHOLESTEROL METABOLISM IN THE RODENT.

A Thesis Submitted for the Degree

of

Doctor of Philosophy

by

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1956.

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GENERAL INTRODUCTION.

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CHOLESTEROL.

History.

Cholesterol was the first sterol to be separated from natural materials. This was achieved in 1769 by Poulletier who prepared it from gall stones. Nevertheless more than one hundred years elapsed before the empirical formula, $C_{27}H_{46}O$, was determined by Reinitzer (1888), and the structural formula remained unknown for many more years. It was not until 1932 that Rosenheim and King, and Wieland and Dane put forward proposals which gave rise to the structure now unanimously accepted. Cholesterol may be systematically named cholest-5-ene-3β-ol.

Distribution.

Cholesterol is found in nature exclusively in the animal kingdom where it appears to be ubiquitously distributed. The following values have been cited by Deuel (1955) with reference to the

young adult male rat; the figures given represent the percentage cholesterol contents on a dry weight basis. Brain, 7.07; lung, 2.45; kidney, 1.94; spleen, 1.59; testes, 1.48; liver, 0.95; thymus, 0.85; heart, 0.66; skeletal muscle, 0.26. The adrenal gland has a very high cholesterol content, being about 21 % on a dry weight basis in the case of the rabbit (Weinhouse and Hirsch, 1940). Friedman, Byers and Michaelis (1950) have reported the cholesterol concentration of rat bile to be 12.7 mg. %.

Cholesterol is found in nature both in the free state as the alcohol and esterified with palmitic, stearic and oleic acids (Deuel, 1951). It has recently been suggested that cholesterol linoleate occurs in blood serum (Michalec, 1956). In the brain, spinal cord, gall stones, red blood corpuscles and usually the bile, the sterol is present exclusively in the free state, while elsewhere both alcohol and esters occur in various proportions.

Absorption.

Investigators in this field have used two

main methods of gauging absorption. In the first of these the amount of cholesterol absorbed is taken to be the difference between that ingested and that excreted. A correction is usually applied for the excretion of endogenous cholesterol but the possibilities of synthesis and degradation in the gut complicate the interpretation of results. In the second method analysis of blood or lymph is carried out, increases in the cholesterol content of these fluids being taken to indicate absorption. In both methods tissue analyses are frequently informative.

It was formerly believed that cholesterol and ostreasterol were the only sterols which were absorbed to any appreciable extent by animals (Cook, 1952) but recently evidence has been obtained which suggests that plant sterols may also be absorbed under suitable dietary conditions (Swell, Boiter, Field and Treadwell, 1956). The extent to which cholesterol is absorbed varies with the species and with the composition of the diet. Thus Cook and Thomson (1951) found that rabbits absorbed cholesterol which was incorporated into the diet much more readily than did rats or guinea pigs.

The exclusion of fat from the diet has been claimed to prevent the absorption of cholesterol in the rat (Cook, 1936) but this has been contradicted by Pihl (1955a). Rabbits maintained on a low fat diet did absorb cholesterol when this was administered orally as a fine aqueous suspension (Popják, 1946). There is general agreement that fat facilitates cholesterol absorption.

Absorption is also influenced by the presence or absence of bile in the intestine. The addition of 0.5 % bile salts to the diet of rats was found to increase absorption (Alfin-Slater, Schotz, Greenberg and Deuel, 1952) and studies with the ^{14}C-labelled sterol have convinced some investigators of the obligatory function of bile in the intestinal absorption of cholesterol in this species (Siperstein, Chaikoff and Reinhardt, 1952). It has been suggested that fat favours absorption by virtue of the increased secretion of bile which it induces (Peterson, Schneour, Peek and Gaffey, 1953) and Deuel (1955) offered the opinion that cholesterol absorption is rendered possible by the hydrotropic action of bile salts, facilitated by the solution of the sterol in fat.

The importance of esterification as a prerequisite for absorption is not yet fully understood. Mueller (1915) detected an increase in the amount of cholesterol present in the chyle of dogs fed either the free sterol or its esters. Evidence for the existence of a reversible cholesterol esterase system in the intestine has accumulated from the laboratories of various investigators (Frölicher and Süllmann, 1934; Nieft and Deuel, 1949; Pihl, 1955b). Swell, Byron and Treadwell (1950) demonstrated the presence of such a system in the intestinal mucosa of the rat and observed that bile salts were necessary to activate the enzyme system. They suggested that the pancreas was the major if not the only source of the enzyme since very little of the latter was found in the mucosa of rats which had been almost completely depancreatized. Nevertheless the pancreas has been shown to be non-essential for cholesterol absorption in the chick (Stamler and Katz, 1951).

Considerable attention has been given to the possibility of decreasing cholesterol absorption, and this has been achieved in the chick (Peterson, 1951), the rat (Ivy, Lin and Karvinen,

1955) and the human (Pollak, 1953) by the simple expedient of including plant sterols in the diet. How the latter interfere with the absorption of cholesterol is not known but Ivy et al. (1955) suggested that soya sterols were effective in this respect in the rat by virtue of their competition with cholesterol for the total sterol absorptive capacity of the intestine.

Transport.

Using dogs, Mueller (1915) established that cholesterol which was absorbed in the intestine, passed into the lymphatic system and could be detected in the thoracic duct. This route has since been shown to account for the absorption of virtually all dietary cholesterol, little or none being transported via the portal venous system (Biggs, Friedman and Byers, 1951; Chaikoff, Bloom, Siperstein, Kiyasu, Reinhardt, Dauben and Eastham, 1952). While in the lymph the cholesterol was shown to be present as finely divided chylomicrons (sometimes considered to be very large low-density lipoproteins), on entering the blood stream these under-

went "changes in number, size and composition" (Byers and Friedman, 1954). This is in accord with the observation made by Pierce (1954) that when lipoproteins obtained from a cholesterol-fed rabbit were injected into a normal rabbit, a serial conversion of these large low-density lipoproteins to smaller higher-density lipoproteins occurred in the recipient. The change was progressive so that even the concentration of the relatively small lipoprotein molecules gradually decreased. Such changes involve a loss of lipid from the molecules as they become smaller and it seems probable that they play a part in normal lipid metabolism.

The plasma lipoprotein patterns of the various species are not identical but this field of investigation remains to be fully explored. Human blood plasma has been the subject of study of many workers and it has been shown that in a fasting plasma sample the cholesterol is present almost entirely as a constituent of the α- and β-lipoproteins (Gurd, Oncley, Edsall and Cohn, 1949). These have been characterized chiefly by their electrophoretic behaviour and their solubilities. In men the former carries about 25 %

and the latter about 75 % of the total plasma cholesterol, while in young women the distribution is slightly more in favour of the α-lipoprotein (Oncley, Gurd and Melin, 1950; Nikkilä, 1953; Barr, 1955). How cholesterol is bound in lipoproteins is not known.

Deposition and Mobilization.

Cholesterol deposition occurs to varying extents in the tissues of different species. Unfortunately, many of the experiments designed to study this subject have involved the feeding of large quantities of the sterol, sometimes to herbivora which would normally ingest a cholesterol-free diet. In the dog the intravenous injection of deuteriocholesterol resulted in an even distribution of the radio-activity between the blood and all organs except the lungs, the liver and the central nervous system. The lungs and the liver contained higher concentrations of radio-active cholesterol than did the blood while the cholesterol of the brain and spinal cord possessed no radio-activity (Bloch, Berg and Rittenberg, 1943). There is some

evidence that in the rat under physiological conditions cholesterol is transferred from the plasma to the kidneys, lungs, spleen, testes and adrenals, all of which are themselves able to synthesize the sterol (Landon and Greenberg, 1954).

The findings of Bloch et al. (1943) mentioned above indicate that, with the exception of that of the central nervous system, tissue cholesterol is in a state of dynamic equilibrium with circulating cholesterol. The fatty livers which develop in rats ingesting a high-cholesterol diet are characterized by their abnormally great content of cholesterol esters (Okey, 1933). The latter are mobilized in common with neutral fat when lipotropic agents such as choline and methionine are administered (Beeston, Channon and Wilkinson, 1935; Channon, Manifold and Platt, 1938).

Biosynthesis.

In the early part of this century it was commonly held that the cholesterol occurring in animal tissue was completely exogenous in origin. This idea seems to have sprung from the belief that

cholesterol and the phyto sterols were isomeric and that it was very unlikely that animals could synthesize such complex molecules. The transition from such mistaken ideas to the present degree of understanding of cholesterol biogenesis has been a gradual one. The main lines along which progress has been and is being made are outlined below.

In 1920 Gamble and Blackfan claimed to have demonstrated the biosynthesis of cholesterol in their balance experiments on human infants. The work of Channon (1925) and of Randles and Knudson (1925) confirmed this claim. These workers observed that rats grew normally and accumulated cholesterol in the tissues while maintained on a cholesterol-free diet.

Virtually nothing was known of the details of cholesterol biosynthesis until isotopic tracer techniques were applied to the problem. Rittenberg and Schoenheimer (1937) reported that deuterium labelled cholesterol could be isolated from mice which had received daily injections of deuterium oxide for a considerable period. From the experimental data these authors concluded that cholesterol must be biosynthesized by the coupling

together of a relatively large number of small molecules. A few years later Bloch and Rittenberg (1942) showed that deuterium labelled acetate could be used for cholesterol biogenesis in the rat and that the isotope entered both the polycyclic nucleus and the iso-octyl side chain of the molecule.

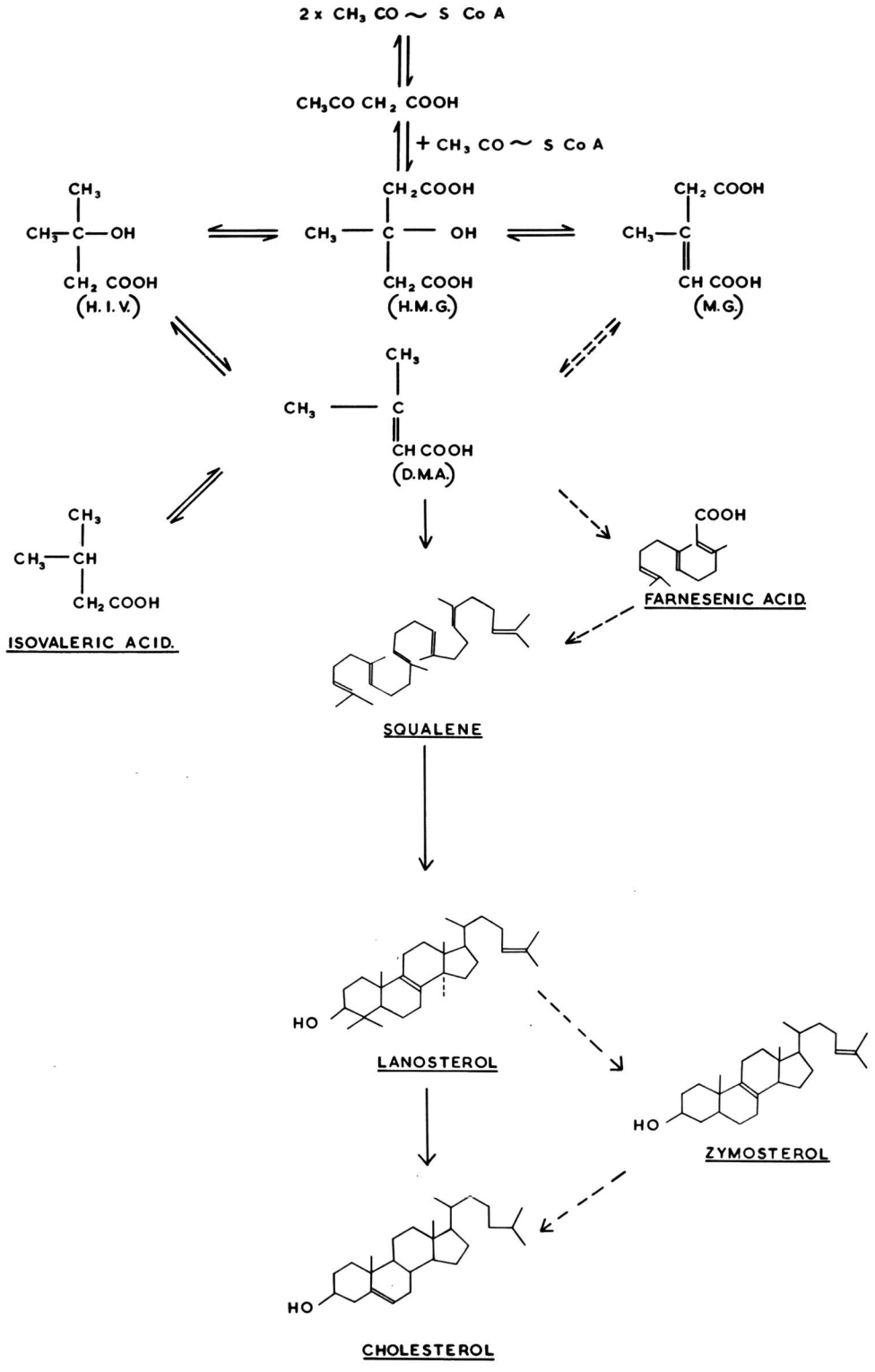
Bloch, Borek and Rittenberg (1946) made an important contribution to investigational technique when they described the in vitro synthesis of cholesterol from (^{13}C)-labelled acetate by surviving liver slices. Isotopic carbon was detected both in the nucleus and in the side chain of the molecule. Subsequent studies have shown that probably all twenty-seven carbon atoms of the sterol molecule are derivable from acetate, and that fifteen of these are supplied by the methyl radical of acetic acid while the remaining twelve derive from the carboxyl group. (Little and Bloch, 1950; Wuersch, Huang and Bloch, 1952; Cornforth, Hunter and Popják, 1953a and b).

The finding that acetate appeared to be the unit from which cholesterol was biosynthesized promoted attempts to correlate coenzyme-A with the

synthetic mechanism (Klein and Lipmann, 1953; Boyd, 1953). These and subsequent studies (Rabinowitz and Gurin, 1954a) have established that coenzyme-A is involved in cholesterol biogenesis, at least in the initial stages. It is difficult to explain the abnormally high rate of hepatic synthesis of cholesterol from acetate which Lata and Anderson (1955) observed in their pantothenate deficient rats.

The investigation of more complex molecules than acetate as possible intermediates on the pathway from the two-carbon compound to the sterol was made practicable, in vitro, by the discoveries that suitably prepared tissue homogenates (Bucher, 1953) and particle-free liver extracts (Rabinowitz and Gurin, 1953) were capable of cholesterol synthesis. It was shown by various groups of workers that such homogenates and extracts could also synthesize from acetate certain branched-chain acids containing five or six carbon atoms, some of which had previously been proposed on theoretical grounds as precursors of cholesterol. Such acids were β-dimethylacrylic acid (Rudney, 1954a; Rabinowitz, 1954), β-hydroxy-β-methylglutaric acid (Rudney,

THE BIOSYNTHESIS OF CHOLESTEROL



SOLID ARROWS INDICATE REACTIONS KNOWN TO OCCUR.
BROKEN ARROWS INDICATE POSSIBLE REACTIONS.
H.I.V. :-β—HYDROXYISOVALERIC ACID.
H.M.G.:-β—HYDROXY-β—METHYLGLUTARIC ACID.
M.G.:- TRANS-β—METHYLGLUTACONIC ACID.
D.M.A.:-β—DIMETHYLACRYLIC ACID.

1954b), trans-β-methylglutaconic acid (Rabinowitz and Gurin, 1954b) and β-hydroxyisovaleric acid (Rabinowitz, 1955). Furthermore, to date, three of these have been shown to act as precursors of cholesterol, without prior breakdown to acetate and acetoacetate, when fed to rats (Bloch, Clark and Harary, 1954). Since β-dimethylacrylic acid was used in cholesterol synthesis *in vivo* two to four times as efficiently as was acetate, while the others were used less efficiently than acetate (Bloch *et al.*, 1954), it would seem most probable that this acid with the isoprenoid structure is a more specific precursor of the sterol than any other simple compound yet investigated. The possible interconversions of these branched-chain acids are shown in the diagrammatic representation of cholesterol biosynthesis which faces page 13.

Information has also been forthcoming with regard to the nature of the more immediate precursors of the complex sterol molecule. As long ago as 1926 Channon proposed the triterpene squalene ($C_{30}H_{50}$) as a precursor on the basis of the increase in liver cholesterol which he observed to follow squalene feeding in the rat. This

suggestion was proved correct by the work of Langdon and Bloch (1953a and b). These workers fed (1 - ^{14}C) - acetate plus non-radioactive squalene to rats and isolated from these animals the pure triterpene which then contained labelled carbon. The radio-active squalene so prepared was converted to cholesterol when fed to mice. The relative specificity of this complex molecule as a cholesterol precursor was indicated by the absence of radio-activity from fatty acids isolated from these animals. The simple acids hitherto identified as cholesterol precursors had always been incorporated into fatty acids to some extent during the experimental procedure.

Popják (1954) has said that the distribution of the carbon atoms of acetic acid in cholesterol is compatible with the hypothesis that the biosynthesis of this sterol involves the condensation of isoprenoid units, with squalene as a possible intermediate. Further, Cornforth and Popják (1954) showed that the entire carbon skeleton of squalene could be biosynthesized from acetate and that the reaction sequence might be as follows:

Acetate + acetate ⟶ acetoacetate $\xrightarrow{+\text{ acetate}}$ β-hydroxy-β-methylglutaric acid ⟶ β-dimethylacrylic acid $\xrightarrow{\times 6}$ squalene.

Recent work has confirmed this scheme (Rudney, 1954a and b; Bloch et al., 1954; Rudney, 1955; Bonner, 1955; Diturit, Cobey, Warms and Gurin, 1955) and it has been suggested that farnesenic acid may lie on the pathway from β-dimethylacrylic acid to squalene (Diturit et al., 1955).

Finally, the cyclization of squalene to cholesterol apparently proceeds via lanosterol (lanost-8: 24-diene-3β-ol). Tchen and Bloch (1955) found that their rat liver preparations could cyclize squalene to lanosterol and demethylate the latter to cholesterol. The findings of Schwenk, Alexander, Fish and Stoudt (1955) suggest that several steroids may be produced by the cyclization of squalene and the subsequent modification of the product. Among these is zymosterol (cholest-8: 24-diene-3β-ol) which could be produced by demethylation of lanosterol, and which could give rise to cholesterol by hydrogenation of one double bond and migration of the other.

The outline of cholesterol biosynthesis given above has been summarized in the diagram facing page 13 , but it must be admitted that certain quantitative isotopic studies of Popják (1954) make the inclusion of squalene in such a key position open to doubt. Further, the branched-chain acids mentioned above can be derived biologically from precursors other than acetate. For example, dehydrogenation of isovaleric acid, an intermediate in leucine metabolism, would yield β-dimethylacrylic acid which in turn could give rise to the other branched-chain acids (Bachawat, Robinson and Coon, 1956).

In conclusion, it must be mentioned that although most studies on cholesterol biogenesis have been made with the aid of liver preparations, synthesis from acetate can occur in many other tissues such as adrenal cortical tissue (Srere, Chaikoff and Dauben, 1948), kidney, testes, intestine and skin (Srere, Chaikoff, Treitman and Burstein, 1950), aorta (Siperstein, Chaikoff and Chernick, 1951) and the ovarian tissues of the laying hen (Popják and Tietz, 1953). Though brain tissue from young rats has the ability to synthesize

cholesterol from acetate, adult brain does not (Waelsch, Sperry and Stoyanoff, 1940; Srere et al., 1950). There is no evidence to suggest that the synthetic pathway in such tissues differs from that followed in liver preparations.

Destruction and Excretion.

Balance experiments have established that in vivo destruction or modification of dietary cholesterol occurs in several species (Dam, 1931; Page and Menschick, 1932; Menschick and Page, 1933; Schoenheimer and Breusch, 1933). Coprosterol, and to a lesser extent dihydrocholesterol, which are isomeric reduction products of cholesterol, have long been recognised as constituents of faeces and Bruger and Winterseel (1929) estimated that in human faeces half the total sterol was coprosterol and the remainder cholesterol. It was natural to suppose that the saturated faecal steroids mentioned above should be derived from cholesterol and many experimental observations upheld this view. For example it was demonstrated that the feeding of a diet rich in cholesterol markedly increased

the faecal excretion of coprosterol in the dog (Dorée and Gardner, 1908) and in the human (Bischoff, 1930).

Such findings predisposed some investigators to believe that coprosterol was the chief excretion product of cholesterol metabolism and this view persisted until comparatively recently (Weinhouse, 1943; Bloch, 1950). However, some of the balance experiments referred to previously had revealed that the feeding of large amounts of cholesterol led to the excretion of compounds which were not precipitable by digitonin and which, consequently, could be neither coprosterol nor dihydrocholesterol (Schoenheimer and Breusch, 1933). Further, it was discovered that cholesterol could be biologically converted to cholic acid (Bloch, Berg and Rittenberg, 1943), progesterone (Bloch, Berg and Rittenberg, 1945), adrenocortical hormones (Zaffaroni, Hechter and Pincus, 1951) and 7-dehydrocholesterol (Glover, Glover and Morton, 1952), and so the existence of degradative routes leading to products other than coprosterol became probable.

Elegant experiments involving the use of cholesterol labelled with isotopic carbon at various

positions in the molecule have since shown that the emphasis on coprosterol as the main excretory product of cholesterol metabolism was entirely misplaced. In one such experiment in which rats received cholesterol labelled at C_4 and C_{26}, either by intravenous injection or orally, extensive oxidation of the iso-octyl side chain to carbon dioxide (labelled) was observed to take place while labelled 'nuclear' carbon appeared almost exclusively in the faeces (Siperstein and Chaikoff, 1952). These authors calculated that not less than 90 % of the labelled 'nuclear' carbon recovered in the faeces was derived from the bile, while not more than 10 % entered the colon by excretion through the intestinal wall. Further, the saponifiable nature of the majority of the nuclear-labelled compounds entering the bile and the faeces and the non-saponifiable nature of those entering the faeces via the intestinal wall led the authors to conclude that only a small fraction of body cholesterol is excreted as such or as coprosterol or dihydrocholesterol. The labelled compounds detected in bile after the absorption of labelled cholesterol were soon identified as bile acids (Bergström, 1952;

Siperstein, Jayko, Chaikoff and Dauben, 1952) and quantitative studies have produced convincing evidence that taurocholic acid is the main excretion product of cholesterol metabolism in the rat (Bergström and Norman, 1953; Siperstein, Harold, Chaikoff and Dauben, 1954), while glycocholic acid is the corresponding product in the human (Siperstein and Murray, 1955).

In conclusion it can be said that the oxidation of the iso-octyl side chain of the cholesterol molecule probably occurs in the liver, since in vitro this tissue has been found to be so much more active than any other in this respect (Meier, Siperstein and Chaikoff, 1952).

Endocrine Influence on Cholesterol Metabolism.

At the present time many aspects of cholesterol metabolism are under investigation and both the academic and the clinical approach are proving successful in elucidating the main rôles and characteristics of the sterol in the animal body in health and disease. The clinical approach has established strong correlations between derangements in cholesterol metabolism and certain pathological condi-

tions. The best known of the latter is coronary atherosclerosis which has been shown to be associated with hypercholesterolaemia in humans (Gertler, Garn and Lerman, 1950), but diabetes mellitus (Rabinowitch, 1929) and states involving derangement of the thyroid gland (Peters and Man, 1950) have also been linked with some abnormalities of cholesterol metabolism. Consideration of the physiological nature of these complaints suggests that the metabolism of cholesterol may be under hormonal control and a considerable weight of experimental evidence supports this idea.

The influences of most of the known hormones on some aspects of cholesterol metabolism have been, or are at present being investigated in several species but most attention has probably been paid to oestrogens in this respect. Various workers have shown that certain oestrogens can bring about a considerable decrease in the concentration of cholesterol in the blood of human patients (Eilert, 1949; Barr, 1953; Oliver and Boyd, 1954; Boyle, 1954). Further, cyclic variations in plasma cholesterol level during the menstrual cycle in normal women have been reported, in which

the lowest concentration of cholesterol coincided with the period at which oestrogen activity is believed to be maximal (Oliver and Boyd, 1953). Such observations as these prompted the investigations to be reported here.

Scope of Study.

In any study of cholesterol metabolism measurements must be made of certain parameters such as the concentration of cholesterol in blood plasma and various tissues, the rate of synthesis of cholesterol in certain tissues, the rate of utilization or degradation of the sterol and the degrees of absorption and excretion of it. Accurate methods are available for the extraction and estimation of cholesterol in animal tissues and fluids, and these procedures present no difficulty. The liver is the site at which most of the circulating cholesterol is synthesized (Gould, Campbell, Taylor, Kelly, Warner and Davis, 1951) and the latter appears to be in equilibrium with hepatic cholesterol. Consequently, the concentration of cholesterol in the liver and the rate of synthesis of the sterol from radio-active precursors by liver slices

in vitro are often determined. Both synthesis of cholesterol from acetate (Srere *et al.*, 1948) and conversion of the latter to steroid hormones (Zaffaroni *et al*, 1951) are known to occur in the adrenal glands which have an extremely high cholesterol content. By virtue of these and other considerations, the adrenals usually merit attention.

Studies of the rate of degradation of cholesterol have not been numerous owing to the difficulties involved in the isolation, characterization and estimation of the products, but these problems are currently being dealt with. Observation of the degrees of absorption and excretion would obviously be of interest in most investigations unless the supply of exogenous cholesterol were particularly small.

In the present study it was decided to investigate some of the effects of oestrogens on cholesterol metabolism in the rodent and also to examine the pregnant and the castrated animal, in both of which, it was felt, the endocrine imbalance

must primarily be one involving the sex hormones. Particular attention was paid to the concentration of circulating cholesterol and the rate of hepatic synthesis from acetate *in vitro*. Rats and rabbits were used in order to make the study a comparative one. In addition, the possibility of variation in the level of serum cholesterol in the rat with advancing age was investigated, partly for its value as a background study against which to view the results of other experiments, and partly for its intrinsic merit.

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NOTE ON METHOD OF PRESENTATION.

This investigation fell into four more or less separate studies and each has been presented as such.

Tables of values have been excluded from the text since it was felt that the experimental results could be more readily appreciated if presented in graphic form. The values relating to the figures in the text and all details of the methods of analysis and measurement used in the investigation have been assigned to the Appendix. The Table of Contents should be consulted for the Appendix page reference.

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PREGNANCY STUDIES.

CHANGES IN THE LEVEL OF CIRCULATING CHOLESTEROL DURING PREGNANCY AND THROUGHOUT THE PUERPERIUM IN THE RAT AND IN THE RABBIT.

INTRODUCTION.

Many studies have been made of the variability of the blood cholesterol concentration under physiological conditions. One of the conditions in which it has been found to vary is the pregnant state. A survey of the literature on this subject revealed that the level of circulating cholesterol has been found to fall, to rise, or to remain unchanged depending on the species.

The rhesus monkey is the only species in which pregnancy has been shown to be accompanied by a mild hypocholesterolaemia (Hartmann and Fleischmann, 1941). A severe hypocholesterolaemia has been observed in the pregnant rabbit and this will be discussed later.

The failure of pregnancy to influence the level of circulating cholesterol in the dog (Baumann and Holly, 1925-26a) and in the rat (Kaufmann and Erdmann, 1932; Rosenman, Byers and Friedman, 1952a) has been reported but the experimental

evidence is open to criticism. In the canine study only three dogs were used, while in both investigations on the rat the cholesterol concentration was determined only once during pregnancy.

A general lipaemia, which included an increase in blood cholesterol, was shown to occur in the fowl which was actively engaged in egg laying, but this was found not to be due to oviposition (Lorenz, Entenman and Chaikoff, 1938b). Boyd and Fellows (1936) reported a rise in the plasma cholesterol content of the guinea pig in the third trimester of pregnancy and in the gravid mare hypercholesterolaemia was established (Brocq-Rousseu, Roussel and Gallot, 1933; Mühlbock, 1937) after the earlier publication of some conflicting results. Since both a rise (Sato, 1937) and a fall (Shope and Gowen, 1928) in serum cholesterol have been described in the pregnant cow it would seem that this species has been inadequately studied. A steady rise in the plasma cholesterol level in human pregnancy has been established recently by Oliver and Boyd (1955a).

As mentioned, a considerable decrease in the cholesterol content of the blood has been ob-

served in the pregnant rabbit (Baumann and Holly, 1925-26a; Boyd, 1936a; Patterson, Hunt and Nicodemus, 1938; Popják, 1946). However the reports on this subject did not concur in either the degree of hypocholesterolaemia or in the time with respect to parturition date at which the lowest concentration of cholesterol was reached.

In view of the paucity of information available on cholesterol metabolism in the pregnant rat and the lack of complete agreement with respect to the pregnant rabbit, it was felt that a detailed study of these rodents would prove worthwhile. Consequently it was decided to determine the plasma or serum cholesterol concentrations in these species at short regular intervals while the animals were still unmated, throughout pregnancy and during the puerperium. (The literature appeared to contain only one description of post-partum studies in the rabbit.) The Schoenheimer-Sperry digitonin method of cholesterol estimation, as modified by Sperry and Webb (1950), was chosen for the serial determinations since it appeared to be one of the most reliable methods available. In addition it was felt that, since it was intended at a later

date to study the influence of oestrogens on cholesterol metabolism in both the rat and the rabbit, the investigation should prove additionally interesting and informative.

MATERIALS AND METHODS.

(a) Rats.

The rats used were young adult albinos of the Wistar strain. All were virgin animals at the outset of the experiment and were mated with adult males of the same strain. Regularly successful mating was obtained by housing together two females and one male, the latter being replaced by other males at two day intervals for six days. The rats were fed an *ad libitum* diet of rat cake (M.R.C. diet No. 41, (Bruce, 1950)) and water throughout the entire experiment.

Twelve rats were bled from the tail vein before mating took place and thereafter at weekly intervals until shortly after the young were weaned. Four virgin females of the same strain and of about the same age served as controls. These

rats were bled at the same intervals as the mated animals for several weeks, but at no time did they come in contact with males. This precaution was taken to avoid any complications which might conceivably have arisen had some of the control rats become even pseudo-pregnant.

Aliquots (0.2 ml.) of individual sera were extracted with a mixture of acetone and ethanol at the boiling point and total cholesterol was determined in these extracts by the Sperry and Webb modification (1950) of the Schoenheimer-Sperry method. The method was scaled down to make practicable the estimation of as little as 20 µg. of cholesterol (Appendix, p. 102 and p. 104). Haematocrit readings were obtained by the micro method of McInroy (1954) at each venisection since changes in the plasma volume alone would influence cholesterol concentration.

(b) Rabbits.

The rabbits used were young adults of the Chinchilla breed. They mated readily with adult males of the same breed. The rabbits were fed an adequate diet of hay, cabbage and bran mash through-

out the entire experiment; they were offered water to drink while the young were being suckled.

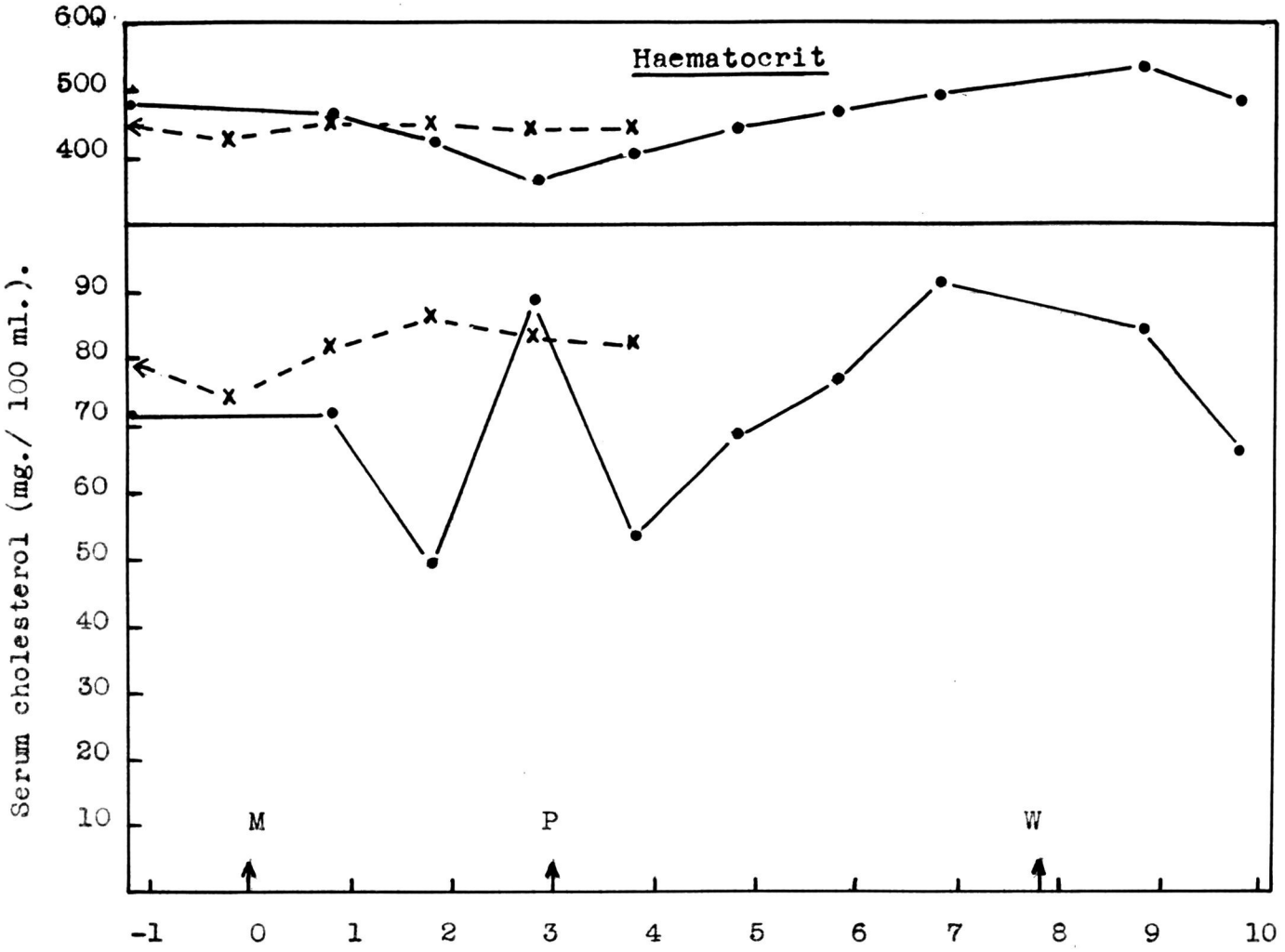
Individual blood samples (3 ml.) were obtained from the marginal ear veins of six females before mating took place and thereafter at regular intervals of three or four days throughout pregnancy and the puerperium. Four of these six rabbits acted as their own controls, blood samples being collected at regular intervals of a few days for several weeks before mating, thus allowing a pre-pregnancy datum line to be drawn.

The method of extraction and the estimation of cholesterol in aliquots (1 ml.) of the rabbit plasma were essentially those applied to the rat blood-serum (Appendix p. 102 and p. 104). Routine micro-haematocrit measurements were made on two of the animals throughout the experiment.

RESULTS.

The fluctuations in the level of serum or plasma cholesterol accompanying pregnancy in the rat and the rabbit are clearly illustrated by

CHANGES IN THE SERUM CHOLESTEROL CONCENTRATION IN THE RAT DURING PREGNANCY AND THROUGHOUT THE PUERPERIUM.



•————•, mated rats (4) all of which littered on the same date.

x— — — —x , unmated control rats.

M, mating date. P, parturition date. W, weaning date.

FIGURE 1.

Figures 1, 2, 3, and 4.

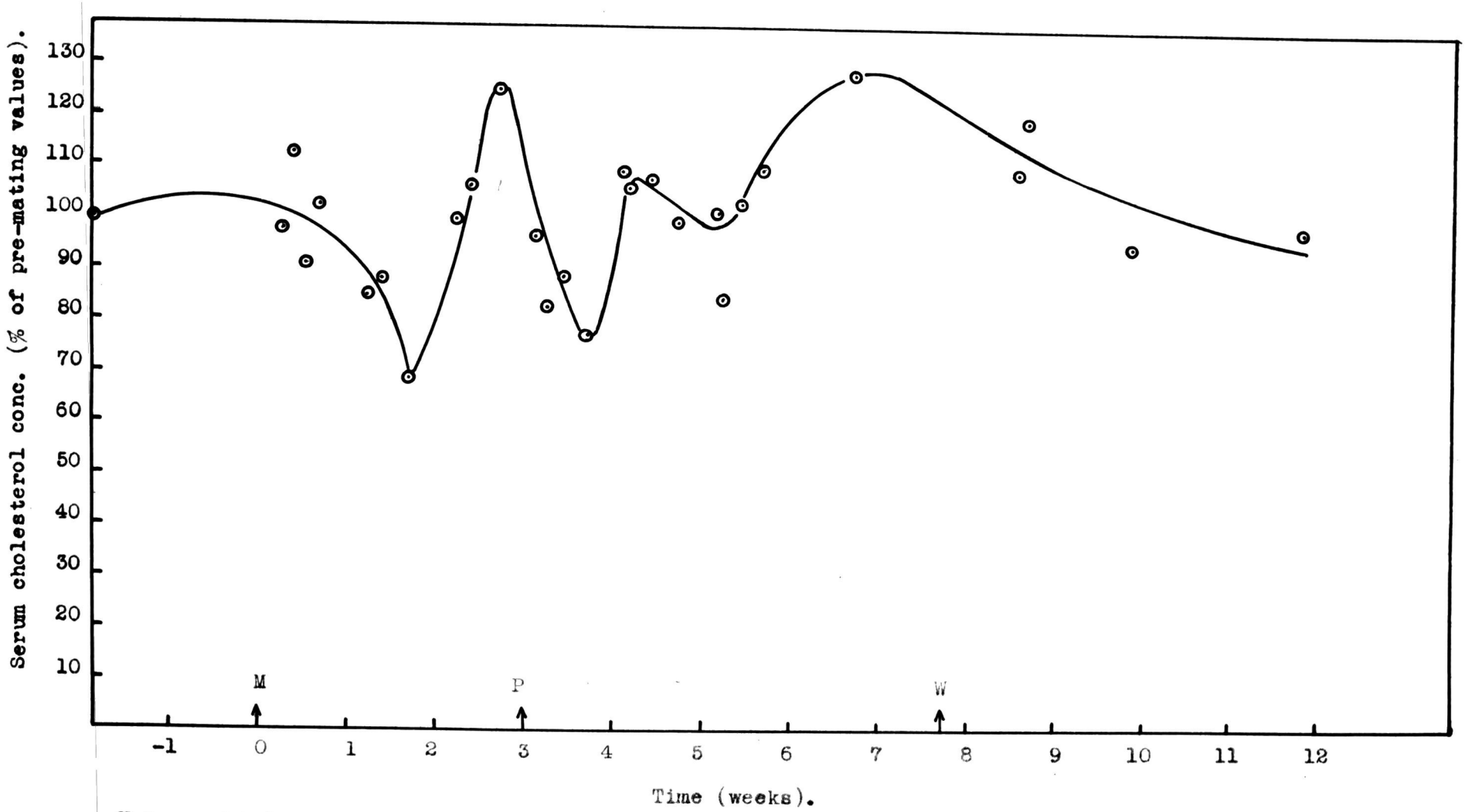
(a) Rats.

In the case of the rat it can be seen (Fig. 1) that in the non-pregnant female the serum cholesterol level was of the order of 70 mg./100 ml. and that this value was maintained during the first trimester of pregnancy. However, during the second trimester the serum cholesterol fell sharply to about 50 mg./ 100 ml., the lowest value recorded for this group of animals. It then rose just before parturition to nearly double the previously mentioned concentration, only to fall again, at, or just after parturition to the low value of approximately 53 mg./ 100 ml.. Thereafter, the serum cholesterol increased gradually until, towards the end of the lactating period, it exceeded the premating level by 20 mg./ 100 ml.. This latter level was regained soon after the weaning of the young.

Haematocrit values varied throughout pregnancy and the puerperium in the manner shown in Figure 1. It can be seen that the observed changes in the serum cholesterol concentration were not merely due to changes in plasma volume.

The parturition dates of eight of the

CHANGES IN THE SERUM CHOLESTEROL CONCENTRATION IN THE RAT DURING PREGNANCY AND THROUGHOUT THE PUERPERIUM.



Values obtained from twelve rats. Each point represents the mean of figures from two to five rats.

M , mating date. P , parturition date. W , weaning date.

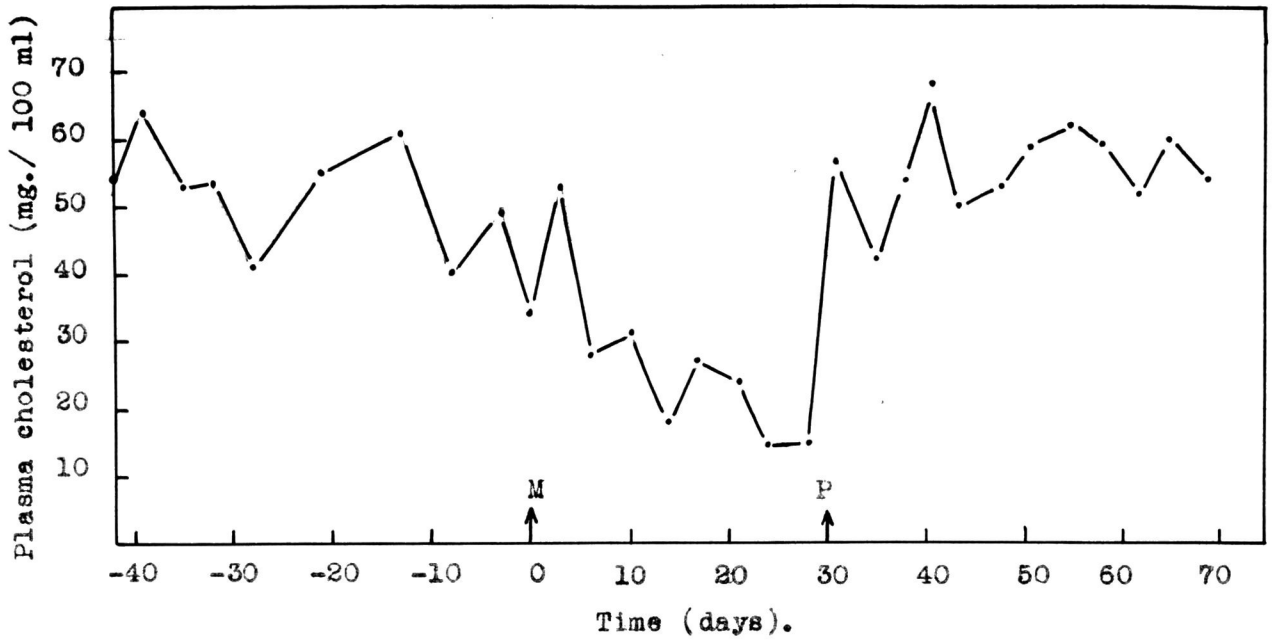
FIGURE 2.

twelve rats did not coincide and consequently the data obtained from all twelve animals was utilized in the following way. The several values of serum cholesterol concentration obtained from each rat were expressed as percentages of the pre-mating concentration in that animal. Each set of percentage values was then plotted against time on a composite graph in such a way that the individual parturition dates coincided on the time scale. This method of presentation (Fig. 2) gave rise to a more comprehensive coverage of the changes in serum cholesterol level than was possible when the data obtained from only four rats was plotted (Fig. 1). It can be seen that the figures are essentially similar. Only one of the rats failed to conform to the general pattern seen here: its serum cholesterol level varied little throughout pregnancy and the puerperium.

(b) Rabbits.

Of the six rabbits used in this work, four were examined at the outset of the study and two more were added considerably later. For reasons

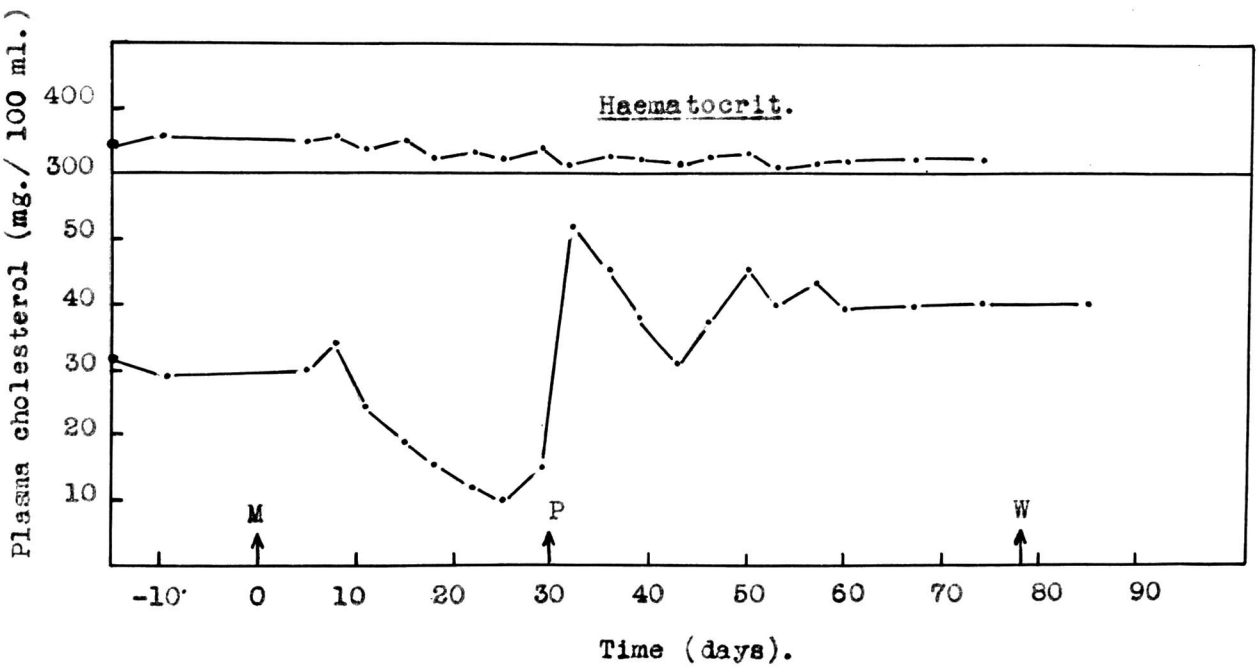
CHANGES IN THE CONCENTRATION OF PLASMA CHOLESTEROL IN THE RABBIT DURING PREGNANCY AND THROUGHOUT THE PUERPERIUM.



Values obtained from rabbit No. 015 of the first group.

M , mating date. P , parturition date.

FIGURE 3.



Mean values obtained from rabbits (2) in the second group.

M , mating date. P , parturition date. W , weaning date.

FIGURE 4.

of convenience which will become apparent shortly, these two small groups have been considered, to some extent, separately.

Figure 3 was prepared from the values of plasma cholesterol of one of the rabbits of the first group of four. This curve is typical of the group and has been produced individually in place of an average curve because the rabbits littered on different days, while blood samples had been taken from all four simultaneously, at the same intervals of time. The completeness of this study was marred by the sudden death of one of the rabbits at its expected parturition date. Until that time its plasma cholesterol concentration had varied in the manner shown for rabbit No. 015 in Figure 3, but just before death a large increase in this value occurred.

The plasma cholesterol concentration of one rabbit varied rather differently from the other three in that it rose considerably just before, or at the beginning of pregnancy and remained elevated during the first trimester. Subsequently it behaved in like manner to the levels in the other

rabbits in all respects.

The results obtained from the second study of two rabbits were in agreement with those of the first. The colorimetric method of estimation of cholesterol used in this work had been improved before the second study was begun and, consequently, the very low levels of plasma cholesterol met with in the third trimester of pregnancy are thought to have been more accurately determined here. Since both rabbits were always bled at the same time and since both littered on the same date, it was possible to construct a curve (Fig. 4) from the average values of the plasma cholesterol concentrations of these animals. The individual values throughout pregnancy and the puerperium were strikingly similar.

Figure 3 shows the considerable variation in plasma cholesterol level which one frequently found in the normal, unmated female rabbit. After mating, the level remained fairly constant throughout the first trimester of pregnancy but thereafter fell gradually, reaching its lowest value in the third trimester, to rise again suddenly at, or just after, parturition to at least the pre-pregnancy

level. During the puerperium the plasma cholesterol concentration fluctuated at, or slightly above, the pre-pregnancy level.

Haematocrit determinations made on two of these rabbits revealed no appreciable change in plasma volume throughout pregnancy and the puerperium.

DISCUSSION.

Only two reports were found in the literature relating to the level of circulating cholesterol in the pregnant rat. Kaufmann and Erdmann (1932) analysed the blood of pregnant rats once, at an unstated time, during the gestation period and, finding normal values, concluded that the blood cholesterol level was normal in the gravid rat. Rosenman *et al.* (1952a) reported no significant change in the concentration of plasma cholesterol throughout pregnancy, but it should be noted that this conclusion was derived from a consideration of very little data. Plasma cholesterol level had in fact only been determined three times - before mating, just prior to mid-pregnancy and twenty-four hours after parturition. The significant rise in

plasma cholesterol concentration twenty-four hours post-partum, observed by these workers, has not been confirmed by the present study.

Unlike the study of the rat, that of the rabbit has confirmed the previously reported findings of other investigators and has contributed to our knowledge of the plasma cholesterol level during the puerperium in this species. Hitherto, only one such investigation had been continued into the post-partum period (Baumann and Holly, 1925-26a).

At the outset of this work considerable attention was given to the fluctuations in the level of plasma cholesterol in individual unmated female rabbits (see Fig. 3). Such variability was not encountered in virgin female rats. Since it had recently been established (Oliver and Boyd, 1953) that there were regular cyclic variations in the plasma cholesterol concentration of young women during the menstrual cycle, an attempt was made to establish a relationship between changes in the plasma cholesterol content of a group of four female rabbits and the presence or absence of oestrus in these animals, as evidenced by the appearance of

the vulva. Though vulvar examination was carried out daily and plasma cholesterol estimations were frequently made, no relationship was established. This is perhaps hardly surprising since it is believed that the domestic rabbit exists on "heat" for months at a time and that the appearance of the vulva is not an absolutely reliable indication of the state in this species (Hammond and Marshall, 1925).

It is interesting to note that although the hypocholesterolaemia of rabbit pregnancy was consistently reported in the literature, the actual concentrations of cholesterol, the percentage decrease, and the period at which the lowest level was reached, were variously reported. Thus Baumann and Holly (1925-26a) found that the blood cholesterol concentration fell from about 90 mg./ 100 ml. in the non-pregnant rabbit to about 50 mg./ 100 ml. before parturition while Patterson et al. (1938) reported a very similar initial figure for whole blood which fell to about 55 mg./ 100 ml. at the end of the second trimester and then rose to 65 mg./100 ml. at term. The plasma cholesterol concentration in the rabbits used by Popják (1946) was approximately

67 mg./ 100 ml. at the time of mating and fell to about 17 mg./ 100 ml. just before parturition. Boyd's figures (1936a), like Popják's, proved to be more like those arising from the present work. In Boyd's animals the initial value was of the order of 54 mg./ 100 ml. of serum and this was found to increase during the first half of pregnancy only to fall to 12 mg./ 100 ml. at term.

The differences in the cholesterol concentrations cited above, and those existing between the older figures and the values encountered by the author, might be explained as follows. The animals used in the several studies were all domesticated rabbits but the breeds were not specified and it is possible that the lipid contents of the blood in different strains are not identical. Further, in two of the early studies (Baumann and Holly and Patterson et al.) blood was analysed, while in the others, and in the investigation reported here, serum or plasma was used. Boyd (1936a) has shown that the cholesterol content of the red cells remains fairly constant during pregnancy in the rabbit and consequently variations in the concentration of cholesterol in whole blood would be less marked than

those observed in serum or plasma. Finally, a diversity of methods of estimating cholesterol was used in the previous studies. In two of these (Baumann and Holly and Patterson et al.) the concentration of the sterol was measured colorimetrically in an unfractionated, unsaponified lipid extract of whole blood. This procedure is known to give misleading results since substances other than cholesterol present in the extract may affect the colour development with the Liebermann-Burchard reagent (Mueller, 1916) and also because cholesterol esters give greater colour intensities with this reagent than do corresponding weights of free cholesterol. (Gardner and Williams, 1921).

Comparative Aspects.

The essential similarity of the changes observed in both the rat and the rabbit are quite striking. While both the extent of the fall in circulating cholesterol and the subsequent increase before parturition serve to distinguish the rat from the rabbit, there are no other major differences. It would appear that these species, together with

the rhesus monkey (Hartmann and Fleischmann, 1941), differ from the other species whose circulating cholesterol has been investigated during pregnancy. Thus a relative hypercholesterolaemia of pregnancy has been observed in the guinea pig (Boyd and Fellows, 1936), the mare (Brocq-Rousseu, et al., 1933; Mühlbock, 1937) and the human (Oliver and Boyd, 1955a) and equivocal results have been reported for the cow (Shope and Gowen, 1928; Sato, 1937) and the dog (Baumann and Holly, 1925-26a).

The rodents used in the present investigation contrast strikingly with the human in whom the concentration of plasma cholesterol has been shown to rise steadily during pregnancy to reach a maximum shortly before the onset of labour, thereafter falling gradually to parturition and throughout the puerperium, only reaching the pre-pregnancy level at about twenty weeks post-partum (Oliver and Boyd, 1955a).

Possible Mechanisms.

The negligible cholesterol content of the rabbit diet makes it unlikely that decreased intes-

tinal absorption of cholesterol was responsible for the observed hypocholesterolaemia, but this possibility cannot be excluded in the case of the rats since their diet contained ca. 0.1 % of cholesterol.

It might be argued that the drop in maternal blood cholesterol level occurred in response to an increasing demand from the developing foeti for this sterol. However, Popják (1950) showed that, at least in the case of the rabbit, this was unlikely since it was calculated that all the foetal cholesterol was synthesized by the foeti themselves.

An increased biliary excretion of cholesterol in pregnancy is also unlikely since this pathway is not normally one which is believed to deplete circulating cholesterol (Byers and Friedman, 1952). Further, Baumann and Holly (1925-26a) found no significant trend in the cholesterol content of the bile of pregnant rabbits while Rosenman et al., (1952a) reported a decreased excretion of biliary cholesterol in the pregnant rat.

Decreased hepatic synthesis of cholesterol might have reduced the blood cholesterol level since most of the circulating cholesterol appears to originate in the liver (Gould, Campbell, Taylor, Kelly,

Warner and Davis, 1951). There is some evidence (Rosenman et al., 1952a) that the liver of the pregnant rat may synthesize cholesterol at a subnormal rate.

Increased deposition and increased degradation of cholesterol in pregnancy must be considered as real possibilities. In regard to the former, Baumann and Holly (1925-26b) pointed out that the increases in cholesterol content of certain tissues (such as the mammary gland) during pregnancy in the rabbit were sufficient to account for the observed decrease in circulating cholesterol. Since, at least in the rat, bile acids are quantitatively the main degradation products of cholesterol metabolism (Sipperstein and Chaikoff, 1955), biliary and faecal analysis would prove informative.

Cholesterol has been identified as a precursor of the adrenocortical hormones (Zaffaroni, Hechter and Pincus, 1951) and of progesterone (Bloch, Berg and Rittenberg, 1945; Saba, Hechter and Stone, 1954); it is possibly also the parent substance of the oestrogens (Heard, Jacobs, O'Donnel, Peron, Saffran, Solomon, Thompson, Willoughby and Yates, 1954). It seems feasible that a sufficient-

ly increased production of these hormones might deplete the cholesterol content of the animal body to a measurable extent. The loss of cholesterol from the adrenals of the pregnant rat (Anderson and Sperry, 1937), the increased levels of circulating 'progestin' in the pregnant rabbit (Zarrow and Neher, 1955) and the depletion of the cholesterol content of the interstitial gland of the rabbit ovary under gonadotrophic stimulation (Claesson, Hillarp and Högberg, 1953), lend some support to this notion. The steroidal excretion during pregnancy in these rodents has not been determined quantitatively.

More direct hormonal influence is also possible. Hypocholesterolaemia has long been known to be present in the hyperthyroid state (Boyd and Connel, 1936) and the increased basal metabolic rate which has been noted in some species during pregnancy (Stewart and Menne, 1933; Javert, 1940) might be thought to implicate the thyroid gland in this effect upon blood cholesterol level. It is difficult to reconcile this plausible theory with the observation of Baumann and Holly (1925-26a) that in thyroidectomized pregnant rabbits there occurred a fall in blood cholesterol which was similar to

that observed in intact pregnant animals.

Boyd (1936a) was able to show a decrease in the serum cholesterol of pseudo-pregnant rabbits in which corpora lutea had developed after mating with castrated bucks. Zarrow and Neher (1955) observed a continuous increase in serum "progestin" during pregnancy in the rabbit. Thus it appears possible that increased secretion of ovarian hormones may be responsible, in some way not yet understood, for the observed alteration in circulating cholesterol level during pregnancy.

SUMMARY.

(i) The serum or plasma cholesterol concentration of twelve female rats and six female rabbits has been measured while the animals were unmated and subsequently throughout pregnancy and the puerperium.

(ii) Both rats and rabbits exhibited a marked fall in the level of circulating cholesterol during pregnancy and a return to approximately normal levels early in the puerperium.

(iii) The rat differed from the rabbit in experiencing the milder hypocholesterolaemia and in the early arrest of the fall in serum cholesterol with a subsequent rise before parturition.

(iv) The results have been compared with those of other workers in this field and some comparative aspects of the subject have been discussed. Mechanisms possibly involved in the production of the observed effects have been discussed.

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A G I N G S T U D I E S.

THE INFLUENCE OF AGING ON THE SERUM CHOLESTEROL LEVEL IN THE RAT.

INTRODUCTION.

In the past many studies have been made in various species, but mostly in the human, of possible changes in the concentration of circulating cholesterol with age. A rapid decrease in the level of plasma cholesterol in the young cockerel was observed during the first week of life but thereafter the level remained fairly constant during the remaining twenty-four weeks of the experimental period (Rodbard, Katz, Bolene, Pick, Lowenthal and Gross, 1951). This is not surprising when one considers the extremely high cholesterol content of the diet of the embryo-chick. Lorenz, Entenman and Chaikoff (1938) reported comparable values of blood cholesterol for immature domestic fowls of both sexes. In the male bird there was no change with age up to the thirty-ninth week of life at least, while in the female the onset of puberty was accompanied by a rise in the blood lipids, including cholesterol.

The establishment of a correlation between hypercholesterolaemia and the clinical evidence of coronary disease in man, and the notion that such disease was characteristically one of old age have combined to promote several studies on the blood cholesterol level of the aging human, from infancy to advanced age. Thus in the human a fairly complete picture is available. The blood cholesterol concentration is at its lowest level in infancy, values of about 50 mg. / 100 ml. of blood having been reported for the new born (Sperry, 1936; Boyd, 1936b, Rafstedt and Swahn, 1954).

Sperry (1936), and others (Senn and McNamara, 1937; Rafstedt and Swahn, 1954) observed a rapid increase during the first few days of life and apparently the adult level of about 200 mg./ 100 ml. of plasma is quickly attained since Hodges, Sperry and Anderson (1943) found no trend or change in value with increasing age from one month to fourteen years of age, the values always being close to the adult mean value. Thomas (1947) reported a similar finding in children aged six to fourteen years. Although the mean adult value is generally agreed to be about 200 mg./ 100 ml. of plasma in America,

where much work has been done on the subject, some investigators have reported a trend towards increasing circulating cholesterol concentration with age in adults. Keys (1952), who has conducted a series of such studies concluded that the serum cholesterol level is at its lowest in early adulthood, rising to a maximum in the sixth decade and falling somewhat in the seventh decade. It would appear that in America at least the mean serum cholesterol concentration of men aged about thirty years is in the region of 200 mg. %, while the value rises to about 250 mg. % in men aged fifty to sixty years. (Keys, Mickelsen, Miller, Hayes and Todd, 1950). The values reported for women of these age groups are comparable to those for men (Barr, 1953).

In a study of spontaneous atherosclerosis in the rabbit, Bragdon (1952) noted a marked lipaemia in young suckling rabbits. The foetal plasma had not been lipaemic and had contained 68 mg. of cholesterol / 100 ml. but the cholesterol concentration rose to 176 mg. / 100 ml. in the twelve hour old offspring and to 295 mg. / 100 ml. three weeks later. During this period the young

rabbits fed exclusively on their dam's milk which contained 50 mg. % of cholesterol, 150 mg. % of phospholipid and 12,000 mg. % of triglyceride. Very soon after the rabbits were weaned to a cube diet prepared from vegetable sources the plasma cholesterol level fell to normal values (ca. 48 mg. / 100 ml.).

Manca (1939) has described a gradual increase in blood cholesterol with age in the guinea-pig.

Conflicting reports have been made with regard to the rat. Rosenman and Shibata (1952) found no regular variation in plasma cholesterol concentration in male rats from the age of six weeks to eleven months but it is of interest that twice during this period the value rose from about 45 mg. / 100 ml. to about 65 mg. / 100 ml. These elevated values were recorded at the ages of six weeks and five months. On the other hand Bargeton, Krumm-Heller and Tricaud (1954) analysed the serum of rats over the age range six to forty weeks and concluded that the serum cholesterol concentration was a linear function of the body weight. The mean value at the age of six weeks (body weight, 125 g.)

was found to be 80 mg. / 100 ml. but the value fell linearly with increasing body weight until the latter equalled 246 g.. This point was reached at an age of eleven and a half weeks and the serum cholesterol was then at its minimum level of 58 mg. / 100 ml. Thereafter the concentration increased linearly with increasing body weight until the experiment was terminated at which time the body weight was 400 g. and the age forty weeks. The serum cholesterol level at this stage was 73 mg. / 100 ml. Working with older rats Chiang (1952) observed "no appreciable difference" in the cholesterol content of blood of animals of both sexes aged from five to thirty-seven months.

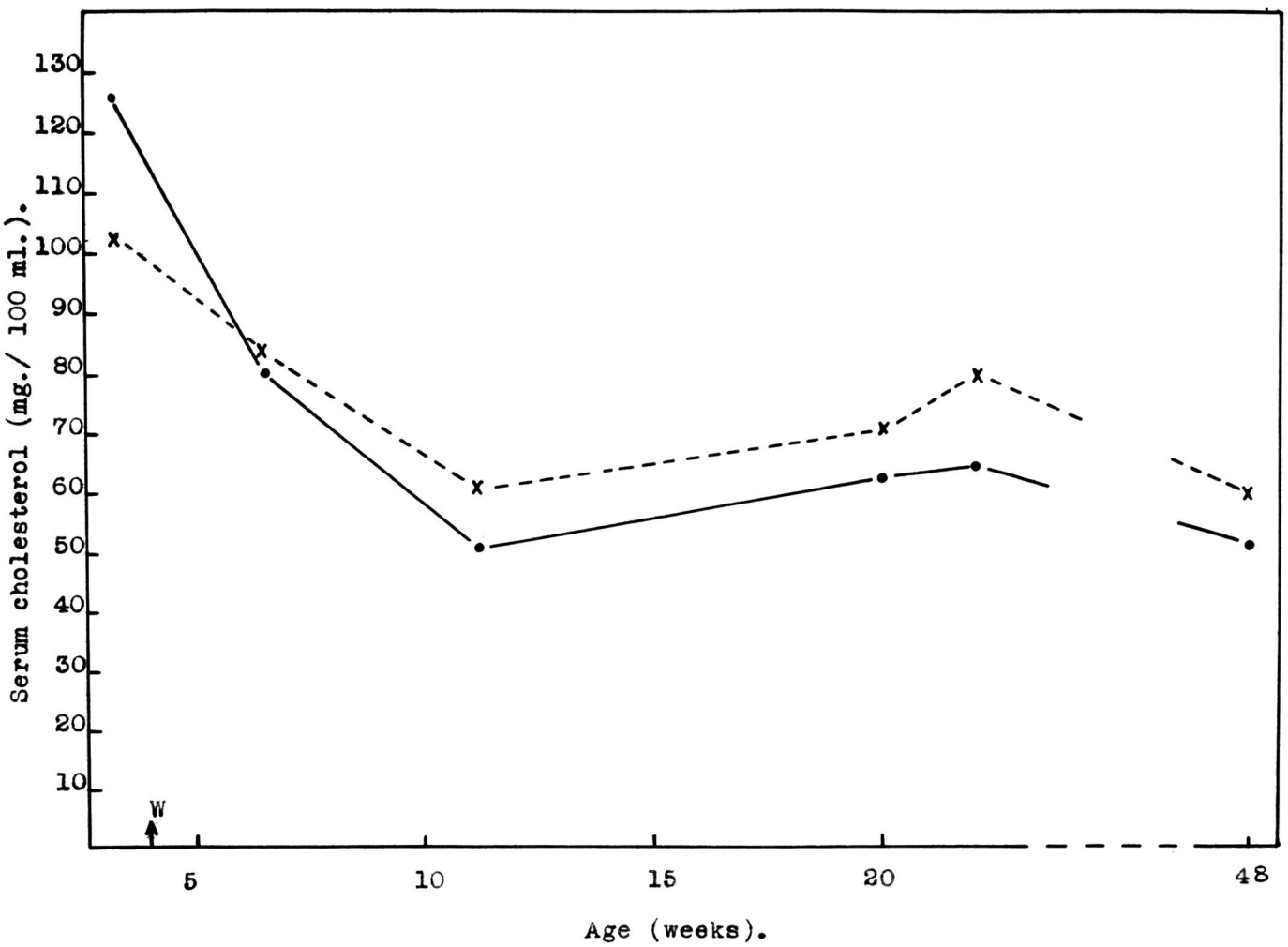
Owing to the prolonged nature of some of some of the proposed studies to be reported later, it was considered desirable to have data on the influence of aging on the serum cholesterol concentration in the rat. Such information would be useful in assessing the significance of results obtained from experiments in which the possible influence of aging might have to be taken into account. The lack of information in the literature relating to very young rats and the findings of others in young

animals mentioned above, led the author to begin the study before the rats were weaned. It was continued until the animals were almost a year old.

MATERIALS AND METHODS.

The rats used were albinos of the Wistar strain. Four of each sex were selected from two litters which had been born at very nearly the same time. The three-week old rats were bled while still feeding at the breast and thereafter at lengthening intervals after weaning until they were eleven months old. The technique of obtaining blood from the tail vein and the extraction of lipid from the serum have been mentioned already and are described in the Appendix (pp.100-2). Serum cholesterol concentration was determined by the Sperry and Webb modification (1950) of the Schoenheimer-Sperry method adapted for micro scale measurements (Appendix, p. 104). Throughout the entire experimental period the rats were offered the standard rat cake diet of the department colony (water plus M.R.C. diet No. 41,(Bruce, 1950)) ad libitum. Since the young rats were not removed from their

THE INFLUENCE OF AGE ON THE SERUM CHOLESTEROL CONCENTRATION IN THE RAT.



Graph of mean values obtained from male (4) and female (4) rats.

•———•, male rats

x------x, female rats

W, weaning date

FIGURE 5.

mothers until they were four weeks old the transition from milk feeding to a rat cake and water diet was a gradual one, but was of course complete at weaning.

RESULTS.

Figure 5 shows the trend of serum cholesterol concentration in the aging rat observed in this study. The changes noted in both sexes were comparable both in degree and direction throughout the period of the experiment. The pre-weaning values which exceeded 100 mg. / 100 ml. proved to be much greater than any other values subsequently determined. After the sharp fall in cholesterol level which occurred in the first two or three months of life, no other striking change was detected. Nevertheless, there was a tendency for the level to increase from a value of less than 65 mg. / 100 ml. at the age of three months to more than 65 mg./ 100 ml. at five months. At the age of eleven months the serum cholesterol concentration was about 55 mg. / 100 ml.

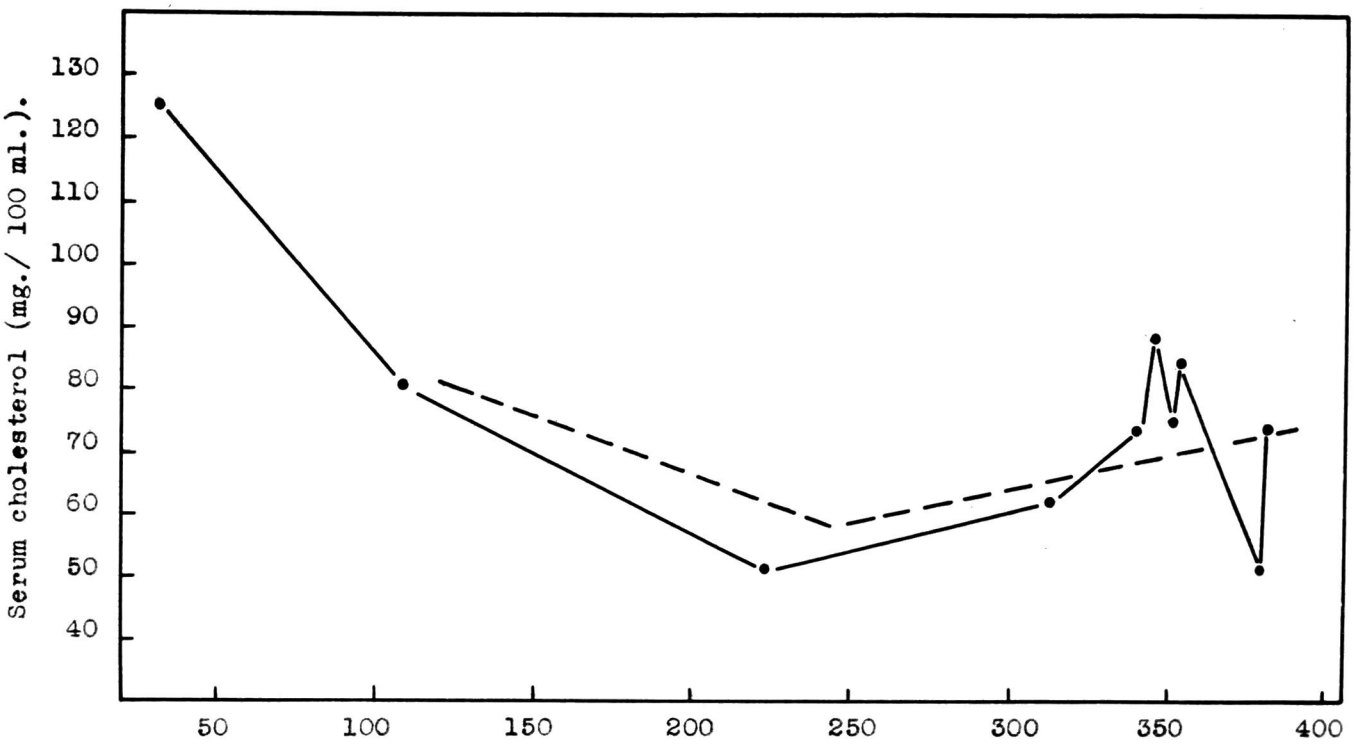
DISCUSSION.

The results of this work illustrate another species difference between the rat and the human with regard to cholesterol metabolism. In the developing offspring of the two species the serum cholesterol levels vary quite differently, rising very rapidly in the human and falling markedly in the rat during the first few weeks of life. It seems probable that such changes are due to dietary conditions. In the human, as the child begins to partake of milk the blood cholesterol level rises steeply (Sperry, 1936). The young domesticated rat gradually at first, and then suddenly, turns from its mother's milk to a rat cake diet and its serum cholesterol falls to about half its pre-weaning value. Similar changes are seen in the young rabbit (Bragdon, 1952). The chick forsakes the high lipid diet of the egg on hatching and its circulating cholesterol concentration falls rapidly (Rodbard, et al., 1951).

It is interesting to note that in a study of the effect of aging on the rate of hepatic syn-

thesis in the male rat, Rosenman and Shibata (1952) recorded figures which indicate that, while the plasma cholesterol concentration was not apparently different in rats aged from six weeks to eleven months, the highest values observed were those of six-week old and five-month old animals in which a level of approximately 64 mg./ 100 ml. was reached in both cases. Between these ages the concentration was about 45 mg./ 100 ml. Reference to Figure 5 will show that in the present study there was a tendency for the serum cholesterol to become somewhat elevated in the five-month old rats. However the two studies are not in agreement in certain other respects. In a later investigation carried out by the author this trend of increasing serum cholesterol was found to persist until the sixth month when a value of 84 mg./ 100 ml. was established. Thereafter the concentration remained fairly constant until the rats were eight months old, after which it fell slowly reaching approximately 70 mg./ 100 ml. by the ninth month. It is not known whether this decline continued until the level of approximately 55 mg./ 100 ml. was reached at eleven months of age. On the other

AGING STUDY: COMPARISON OF RESULTS WITH THOSE OF BARGETON, ET AL. (1954).



Variation in serum cholesterol concentration with body weight in the male rat.

• ——— • , values obtained by the author in this and a subsequent investigation.

— — — — , values obtained by Bargeton, et al.

FIGURE 5a

hand the figures of Rosenman and Shibata show that the increased concentration of plasma cholesterol observed in their five-month old rats (64 mg./ 100 ml.) was not maintained during the next few months. In the seventh month a level of 47 mg./ 100 ml. was determined and a very similar figure was obtained for eleven-month old animals.

While this work was in progress, Bargeton et al. (1954) published results of a similar study in the male rat. As has been mentioned earlier, these workers discovered a linear relationship between the serum cholesterol concentration and the body weight of the animal. The relatively small number of measurements made by the present author on rats during the first few months of life precludes direct comparison of the results reported here with those of the French workers, but Figure 5a (facing p. 56) has been constructed to show that they are not in complete agreement. It can be seen that the trends were similar in both investigations until the rats attained a body weight of some 340 g., after which, in the rats used by Bargeton et al., the level of circulating cholesterol continued to rise regularly with increasing body

weight while in the animals used by the present author no such relationship could be established.

No explanation which would reconcile these conflicting observations suggests itself at present, though it is possible that the differences in behaviour in the three groups of rats discussed above are traceable to their particular breeds. Rosenman and Shibata used rats of the Long-Evans strain while animals of the Wistar strain were used in the investigation reported here. The type of rat used by the French workers was not stated.

It follows from the findings presented here that in any experiment on rats involving the serial measurement of blood cholesterol, concurrent determinations on control animals of the same age should be performed. This would be of particular importance in work in which very young rats were employed.

SUMMARY.

(1) Serial determinations of the concentration of serum cholesterol in the aging albino

rat have been made on animals of both sexes from the age of three weeks to eleven months. No sex difference with respect to changes in cholesterol level was observed.

(ii) The highest cholesterol concentration occurred in the three-week old rat, in which it exceeded 100 mg./ 100 ml. of serum. By the time the rat was three months old this value had halved but thereafter tended to increase again throughout the next few months, reaching about 70 mg./ 100 ml. by the fifth month and probably about 80 mg./ 100 ml. by the sixth. By the eleventh month the level had fallen to around 55 mg./ 100 ml.

(iii) The results of this investigation do not completely agree with the findings of other workers in this field. The degrees of agreement and disagreement of the different studies have been discussed.

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CASTRATION STUDIES.

THE INFLUENCE OF CASTRATION ON THE SERUM CHOLESTEROL LEVEL IN THE RAT.

INTRODUCTION.

Little attention has been given to the possible influences of the testes and the ovaries on cholesterol metabolism.

However, there are numerous references in the literature to the effects of sex hormones and chemically related substances on various aspects of the metabolism of cholesterol. Thus, oestrogens have been shown to produce hypocholesterolaemia in rats (Levin, 1945; Pekkarinen, Kerppipola and Petro, 1952), guinea pigs (Pekkarinen *et al.*, 1952), men (Barr, 1953; Boyle, 1954; Oliver and Boyd, 1954) and women (Eilert, 1949 and 1953), and hypercholesterolaemia in fowls (Lorenz, Chaikoff and Entenman, 1938; Horlick and Katz, 1948). Both an increase (Mori and Reiss, 1928) and a decrease (Pekkarinen, *et al.*, 1952) in circulating cholesterol have been reported in oestrogen treated rabbits. Depletion of liver total lipid (György, Rose and Shipley, 1949) and of adrenal cholesterol

(Vogt, 1945; Levin, 1945) have followed oestrogen administration in rats, while the development of fatty livers has been observed in fowls treated similarly (Musso and Forti, 1953).

Androgens, on the other hand, have not been found to influence cholesterol metabolism so strikingly. They have been shown to favour hypercholesterolaemia in men (Barr, 1953; Oliver and Boyd, 1955b and 1956) but no effects have been reported in rabbits (Ludden, Bruger and Wright, 1941), dogs (Kochakian, MacLachlan and McEwan, 1938) and birds (Riddle, 1947). Simonnet, Thieblot and Segal (1951) observed considerable loss of adrenal cholesterol in rats treated with testosterone propionate.

In the few species in which the effects of castration on cholesterol metabolism have been investigated, equivocal results have sometimes been obtained. In men, the observations are conflicting. McCullagh and Renshaw (1934), in a study of castrated men aged thirty-three to sixty-five years, stated that the blood cholesterol "showed a tendency to be increased". In a series of long-term observations of forty male castrates Teilum (1937) found a defi-

nite increase in serum cholesterol six months after castration and subsequently a further rise during at least the next four years. The results of more recent investigations have contradicted these findings. Thus it was claimed that though the serum lipid pattern in castrated males was higher than that of normal men, it was not changed to statistically significant degrees (Gertler, Hudson and Jost, 1953). Likewise Hamilton, Bunch, Mestler and Imagawa (1956) found that the serum cholesterol concentrations of young adult male castrates did not differ significantly from the pre-castration values over a period of more than four years.

Tanzi (1938) observed an elevation of blood cholesterol in the orchiectomized chick. Blinoff (1930) reported no change in the serum cholesterol of the dog after castration. Bruger et al. (1943) concluded that castration per se did not alter the blood cholesterol of young female rabbits, but it should be noted that this conclusion was drawn from the study of only three animals over a short period of time. The literature appeared to contain no reference to the effect of castration on the circulating cholesterol in the rat, but castration was

found to be without effect on adrenal cholesterol in the male of this species (Simonnet, et al.,1951).

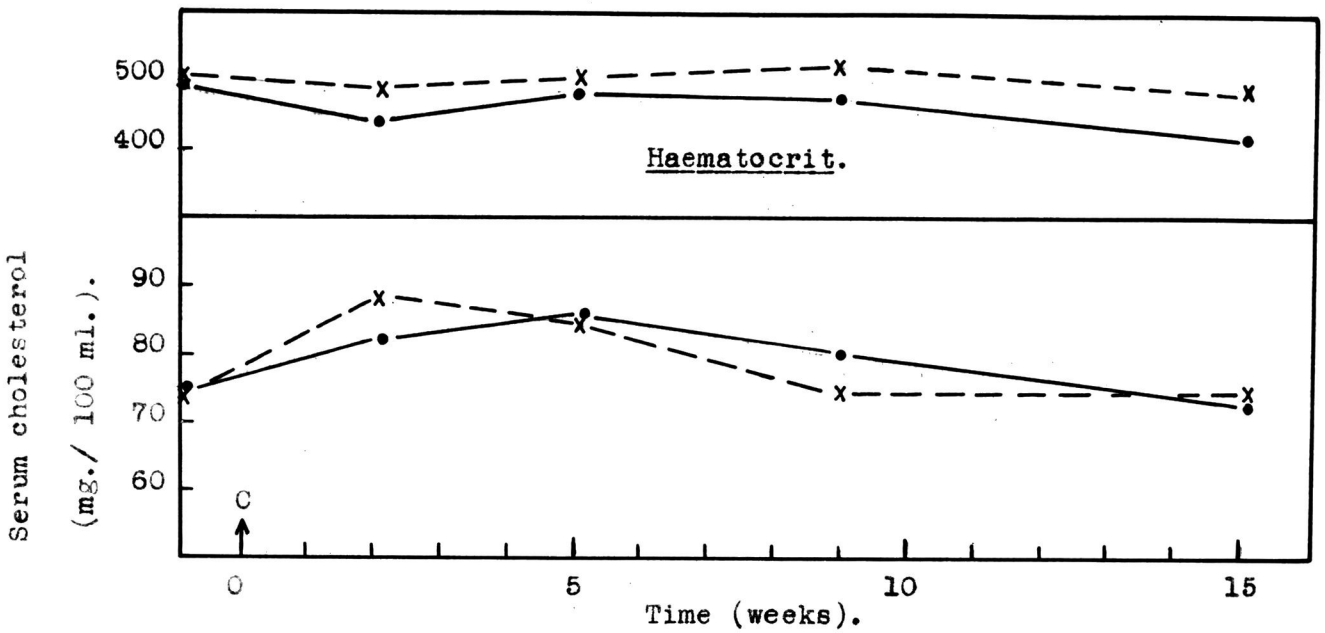
When the present series of investigations was embarked upon it seemed desirable to include a study of the effects of removing the glands principally involved in the endogenous production of the sex hormones since the bias of the work in general was towards the influence of oestrogens on cholesterol metabolism.

MATERIALS AND METHODS.

Twenty, five-month old albino rats of the Wistar strain, ten male and ten female, were selected for this experiment. Five males and five females were castrated under light ether anaesthesia, the former by the usual ventral approach through the skin of the scrotum and the latter by the standard dorsal approach procedure. Recovery appeared to be complete in every case, and the rats showed no sign of ill health at any stage in the period of study.

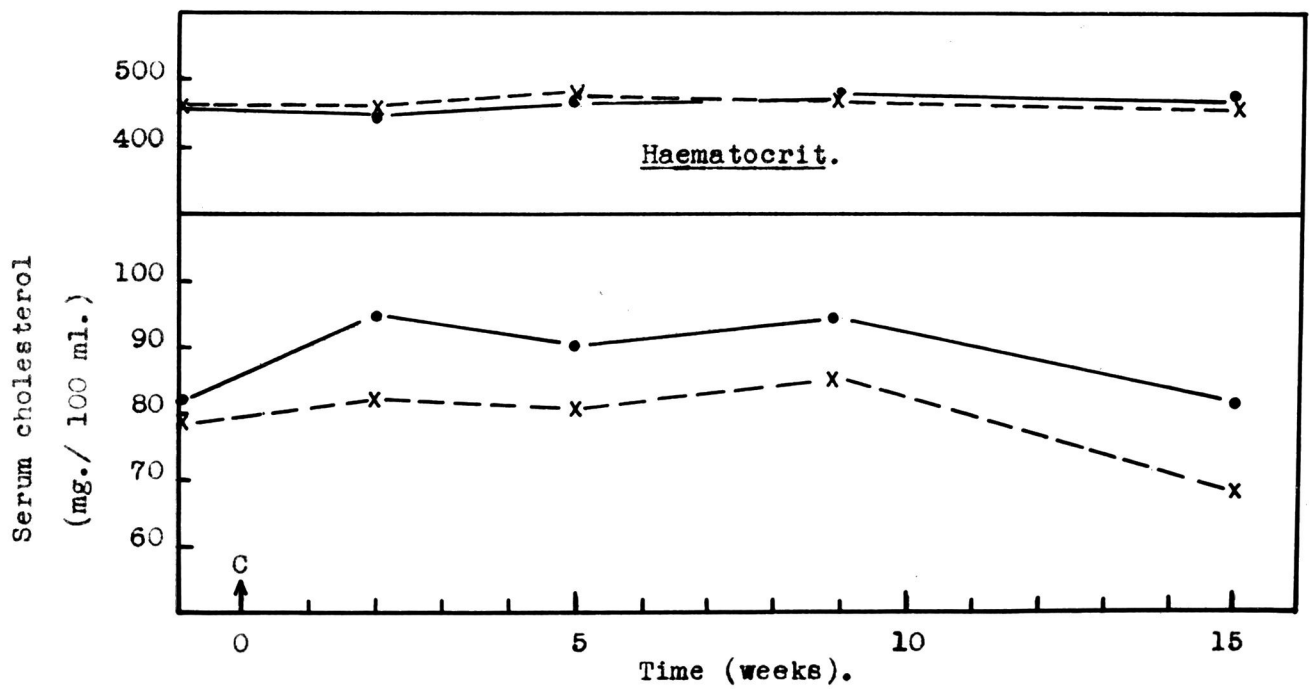
Five animals of each sex were maintained as controls. All the rats were bled from the tail

THE EFFECT OF CASTRATION ON THE SERUM CHOLESTEROL LEVEL IN THE RAT.



Male rats. C, castration date (age, 5 months).
•———• castrates (5). x- - - -x , intact controls (5).

FIGURE 6.



Female rats. C, castration date (age, 5 months).
•———• castrates (4). x———x , intact controls (5).

FIGURE 7.

vein before castration and thereafter at intervals over a period of about four months. The rats were weighed each time before bleeding. Cholesterol was estimated in acetone-ethanol extracts of serum by the Sperry amd Webb modification (1950) of the Schoenheimer-Sperry method, adapted for micro-scale determinations (Appendix, pp. 102 and 104). Micro haematocrit determinations were made on the blood samples, according to the technique of McInroy (1954), in order that any changes in cholesterol concentration attributable to alterations in plasma volume alone might be recognised as such. All the animals were maintained under identical conditions and were offered an adequate diet of rat cake (M.R.C. diet No. 41 (Bruce, 1950)) and water, ad libitum, throughout the entire experiment.

RESULTS AND DISCUSSION.

In Figures 6 and 7, the fluctuation of serum cholesterol level observed in the castrated rat has been compared with that seen in the intact animal. It can be seen that castration was with-

out effect in the male, while a slight increase in circulating cholesterol apparently followed castration in the female. The haematocrit value was unaffected by castration in both sexes.

Inasmuch as the sex hormones have been shown to influence some aspects of cholesterol metabolism in several species, commonly causing variations in the level of circulating cholesterol (Lorenz et al., 1938a; Simonnet et al., 1951; Levin, 1945; Pekkarinen et al., 1952; Barr, 1953), the results of this work are unexpected. The complete removal of the organs chiefly responsible for the production of androgens and oestrogens might have been thought to give rise to metabolic disturbances which would have manifested themselves in changes in the concentration of serum cholesterol in the castrated animals. The lack of response was not due to the growth of accessory gonadal tissue since this was found to be entirely absent at autopsy. It is possible that the removal of the gonads was compensated by increased secretion of androgens and oestrogens by the adrenals. However, in a small group of adrenalectomized castrated male rats the author noted no change in the serum cholesterol

concentration during the four weeks in which they were under observation.

The lack of effect of castration in the rat reported here is in accord with similar findings in the rabbit (Bruger et al., 1943) and the dog (Blinoff, 1930). The slight change observed here in the castrated female rat and the absence of any such change in the castrated male may be related to the greater susceptibility of cholesterol metabolism to the influence of oestrogens as opposed to that of androgens.

SUMMARY.

Serum cholesterol concentration has been determined in castrated and intact male and female rats at intervals over a period of about four months. No marked difference was observed between the castrated and the intact animals.

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OESTROGEN STUDIES.

THE EFFECTS OF CERTAIN OESTROGENS ON SOME ASPECTS OF CHOLESTEROL METABOLISM IN THE RABBIT AND THE RAT.

INTRODUCTION.

The synthetic oestrogens comprise a considerable number of laboratory prepared chemical compounds which imitate to varying degrees the actions of naturally occurring oestrogens in the living animal. A variety of metabolic effects, which include many on lipid metabolism, have been observed on administering both synthetic and natural oestrogens to certain species. Thus a marked lipaemia, involving increases in all the blood lipids, and accumulation of body and depot fat have been reported in fowls which were given natural or synthetic oestrogens (Lorenz et al., 1938a; Horlick and Katz, 1948). A striking fall in the level of circulating cholesterol in the rabbit and, to a lesser extent, in the guinea pig was found to follow injections of diethylstilboestrol in these species (Pekkarinen et al., 1952), although a preparation of natural oestrogens had previously been

shown to induce a considerable increase in blood cholesterol concentration when administered to rabbits (Mori and Reiss, 1928). Many groups of workers have investigated the effect of oestrogens in lowering the level of circulating cholesterol in the human (Eilert, 1949; Barr, 1953; Oliver and Boyd, 1954). As is usually the case, the rat has been the subject of many experiments in this field. Levin (1945) showed that diethylstilboestrol induced a rapid loss of adrenal and serum cholesterol in the adult male rat and that the low levels attained persisted as long as the oestrogen was administered. György et al. (1949), using rats in which 'fatty' livers had been developed by the feeding of an alipotropic high-fat diet, found that administration of ethinyloestradiol significantly reduced liver lipid. This lipotropic effect of oestrogens was further exemplified by Shipley, Chudzik, György and Rose (1950), who reported that the subcutaneous implantation of oestrone pellets prevented the development of 'fatty' livers in castrated female rats. It is of interest to note that this preventative action was lost when the oestrone was implanted in the spleen. From this

site it would drain directly into the liver and suffer inactivation there.

Oestrogens have also been shown to influence carbohydrate metabolism. In the rat, oestrogen administration has generally resulted in accumulation of glycogen in the liver. (Janes and Nelson, 1940; Griffiths, Marks and Young, 1941; Teague, 1942). Since the work of Haugaard and Stadie (1952) suggested a relationship between hepatic glycogen content and hepatic synthesis of lipid, it seems possible that the lipotropic action of oestrogens on the one hand and their influence on glycogen metabolism on the other might be related.

Studies of the adrenal glands may have provided clues to the mechanism of the lipotropic action of oestrogens. Vogt (1945) observed marked depletion of adrenal cholesterol during hexoestrol administration in the rat. Later (1955) she showed that this effect was accompanied by a considerable decrease in corticosterone secretion. These observations suggested that hexoestrol might have exerted an inhibitory effect on cholesterologenesis in the adrenal gland and that the treated rats were probably in a state of adrenal insufficiency.

With such considerations in mind the investigation of the effects of certain oestrogens on some aspects of cholesterol metabolism in the rabbit and the rat was undertaken as follows. The influence of ethinyloestradiol (administered orally) and of hexoestrol (administered parenterally) on the level of plasma cholesterol in the male rabbit was observed. Ethinyloestradiol, hexoestrol, oestrone, oestradiol and oestriol were administered to male rats by subcutaneous injection (ethinyloestradiol was given orally at one dosage level) and the cholesterol concentrations of the serum, the liver and the adrenal glands were determined. In addition, the rates of hepatic synthesis of cholesterol from acetate in vitro and, in certain cases, the liver glycogen concentrations were estimated. The effect of hexoestrol on the serum cholesterol concentration of the adrenalectomized male rat was also studied.

The oestrogen dosages employed in this investigation were of necessity chosen in a rather arbitrary manner, notwithstanding attempts to relate the doses of these substances to their relative oestrogenic potencies and to their relative effi-

cacies as hypocholestrolaemic agents. Such attempts had often to be abandoned because of the lack of reliable data on these subjects with respect to rodents.

MATERIALS AND METHODS.

Rabbits.

Young healthy adult buck rabbits of the Chinchilla breed were used in this experiment. Their diet was the normal one of the department colony consisting of hay, cabbage and bran-mash plus, occasionally, a little turnip. No pair feeding was attempted, but in practice all the rabbits consumed diets which were very similar in quantity and quality.

The animals were bled from the marginal ear vein at intervals before, during and after oestrogen treatment by a technique described in the Appendix (p. 101). Plasma cholesterol concentration was measured by the Sperry and Webb modification (1950) of the Schoenheimer and Sperry method, adapted for micro scale determinations. This adaptation and

the extraction procedure to which the plasma was subjected have been described in the Appendix (pp. 104 and 102). Micro haematocrit readings (McInroy, 1954) were made at each venisection in order that changes in cholesterol concentration due merely to alterations in plasma volume might be recognised as such.

Ethinyloestradiol (British Schering) was administered orally to a group of six rabbits in increasing dosage from an initial level of 7 µg./ kg./ day to a final level of 240 µg./ kg./ day over a total period of about five months. The oestrogen was added to the bran mash, which was fed thrice weekly, in the form of an aqueous suspension, as a fine powder, or as an aqueous-alcoholic solution (0.5 ml.) depending on the quantity required to be added. As a rule the animals consumed the mash readily.

Hexoestrol (B.D.H.) was administered subcutaneously in propylene glycol solution to four rabbits at two dosage levels, viz. at 180 µg./ kg./ day for 15 days and then at 360 µg./kg./ day for 25 days. Two rabbits which acted as controls received daily injections of the vehicle only.

Rats.

Immature male albino rats of the Wistar strain, which were aged usually about five weeks at the outset of the experiment, were employed in this study. Except in a few cases which will be specified, the rats were fed the normal diet of the colony, viz. rat cake (M.R.C. diet No. 41 (Bruce, 1950)) and water, ad libitum. In every experiment but one the oestrogen was administered by subcutaneous injection of a propylene glycol solution, while control rats received injections of the vehicle only. In one experiment ethinyl oestradiol was administered orally by feeding a stiff mash of rat cake powder and water to which had been added a solution of the oestrogen in aqueous glycerol. Except in the case of the experiments involving adrenalectomized animals the analytical procedure was as follows.

The rats were weighed daily. As a rule, serum samples obtained from blood drawn from the tail vein were analysed for cholesterol before, during and at the termination of the experiment. Haematocrit values (McInroy, 1954) were recorded in certain experiments. At the end of the experiment

the rats were sacrificed by severing the cervical vertebrae with bone forceps and the livers, adrenals and, in some cases, the pituitaries were removed and weighed. The rate at which liver slices could incorporate (1 - ^{14}C) - acetate into cholesterol was measured (Boyd, to be published, see Appendix, p. 110). The cholesterol contents of the livers and adrenals were determined and in certain experiments total liver glycogen plus glucose (expressed as glycogen) was estimated by a modification of the anthrone method described by van der Vies (1954) (Appendix, pp. 103, 104 and 108).

As in the rabbit work described above, cholesterol was always estimated by the Sperry and Webb modification (1950) of the Schoenheimer and Sperry method, adapted for micro scale determinations.

Ethinyloestradiol (British Schering) was administered in five experiments at several dosage levels of from 250 µg./ kg./ day to 2600 µg./ kg./ day for periods ranging from eleven to twenty-six days. At each dosage level three to six rats were used with an equal number of control animals, in each case.

Hexoestrol (B.D.H.) was administered to intact rats at two dosage levels, viz. 800 µg./ kg./ day for six days and 850 µg./ kg./ day for fifteen days. In each case four experimental animals and four controls were used.

In two experiments hexoestrol was administered to adrenalectomized rats. In the first of these five rats were subjected to bilateral adrenalectomy by the dorsal route. Twenty-four hours later injections of hexoestrol were begun on three of these at a dosage level of 540 µg./ kg./ day for six days. The other two adrenalectomized rats acted as controls and received daily injections of the vehicle, propylene glycol, only. Three intact untreated rats constituted a second control group.

In the second experiment of this kind eleven nine-week old rats were adrenalectomized. Two weeks later a course of injections of hexoestrol was begun on six of these at a dosage level of 400 µg./ kg./ day for six days. The remaining five rats acted as controls and were given daily injections of the vehicle only. In both experiments all the rats were given physiological saline instead of water to drink from the date of adrenal-

ectomy until the termination of th experiment. The rats were weighed daily. In the second experiment the control rats were offered only as much rat cake per day as had been consumed by the hexoestrol treated rats on the previous day. In both experiments all the rats were bled from the tail vein while still intact and untreated and again at the end of the experiment. Serum cholesterol concentration and haematocrit value were determined from those blood samples. These were the only measurements, apart from those of body weight, made on the animals.

Naturally occurring oestrogens were also investigated with respect to their influence on certain aspects of cholesterol metabolism. Sixteen rats were divided into four groups of equal numbers. One group received injections of oestrone (Ciba) at dosage levels of 3.3 mg./ kg./ day for sixteen days and then 6.4 mg./ kg./ day for a further fifteen days. A second group received oestradiol (Ciba) at a dosage level of 1.9 mg./ kg. / day for sixteen days followed by 3.6 mg./ kg./ day for fifteen days. The third group was given

oestriol [1] at dosage levels of 4.7 mg./ kg./ day for sixteen days and then 9.1 mg./ kg./ day for fifteen days. The animals of the fourth group acted as controls and received daily injections of propylene glycol. Each group was given an amount of rat cake per day which was equal to the smallest weight of cake consumed by any one of the groups on the previous day.

RESULTS.

Rabbits.

The effects of ethinyloestradiol and of hexoestrol on the level of plasma cholesterol in the adult buck rabbit are illustrated in Figures 8 and 9. Administration of both oestrogens was associated with a marked increase in the plasma cholesterol concentration and a decrease in haematocrit value. The decrease in packed cell volume indicated that the increase in plasma cholesterol concentration was not due to haemo-concentration. No significant change in body

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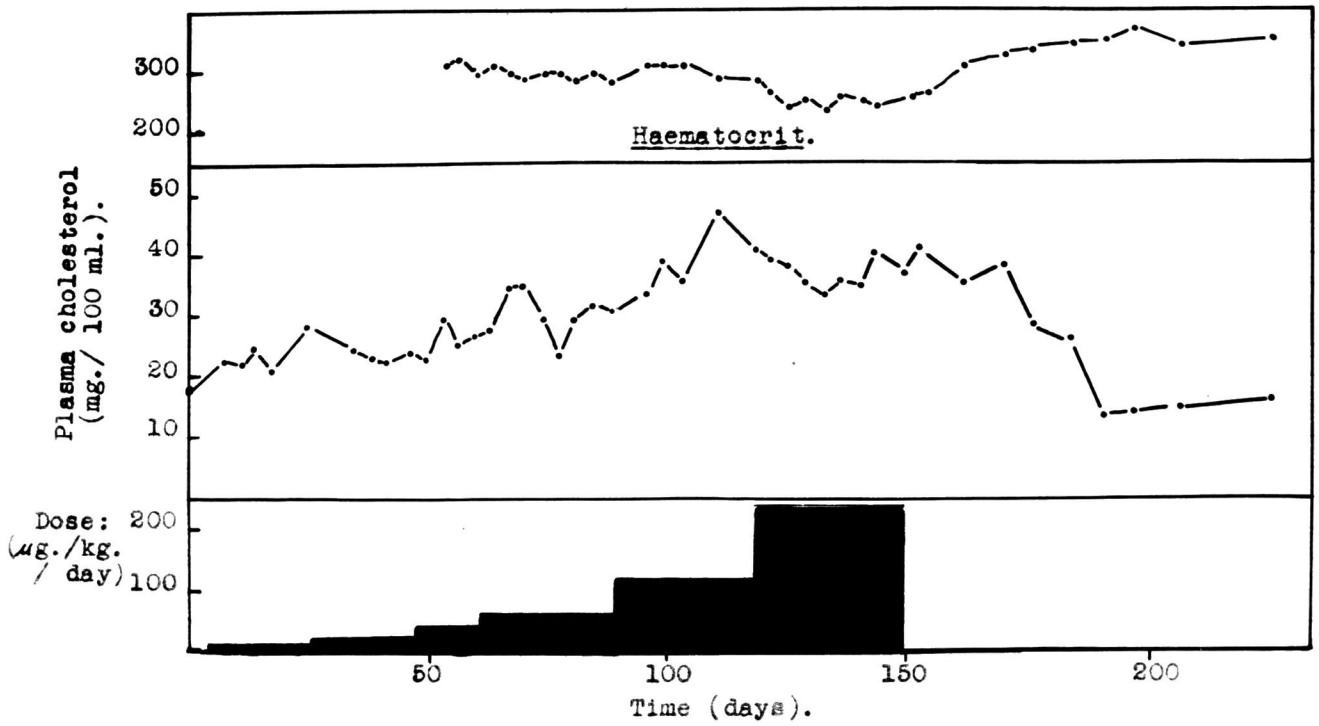
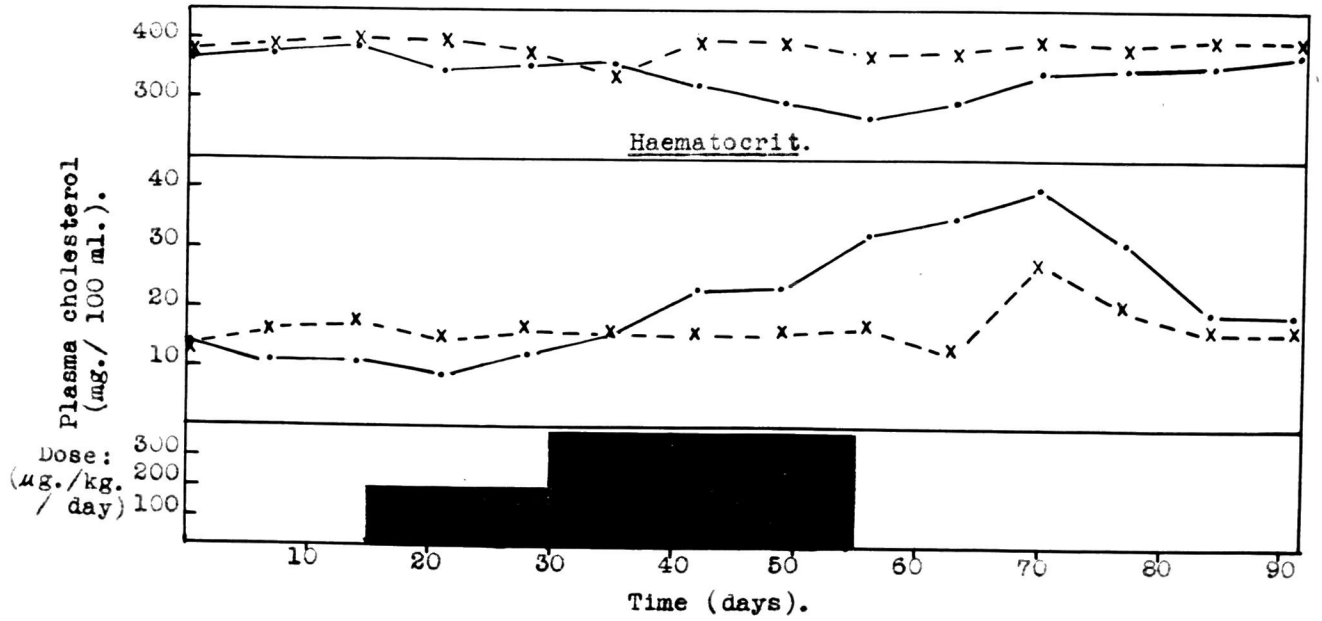
1. Kindly supplied by Prof. G.F.Marrian.

CHANGES IN THE PLASMA CHOLESTEROL CONCENTRATION OF BUCK RABBITS RECEIVING OESTROGENS.



Effect of ethinyl oestradiol on buck rabbits (6).

FIGURE 8.



Effect of hexoestrol on buck rabbits.

•———•, hexoestrol treated (4). x- - - - -x, propylene glycol treated (2).

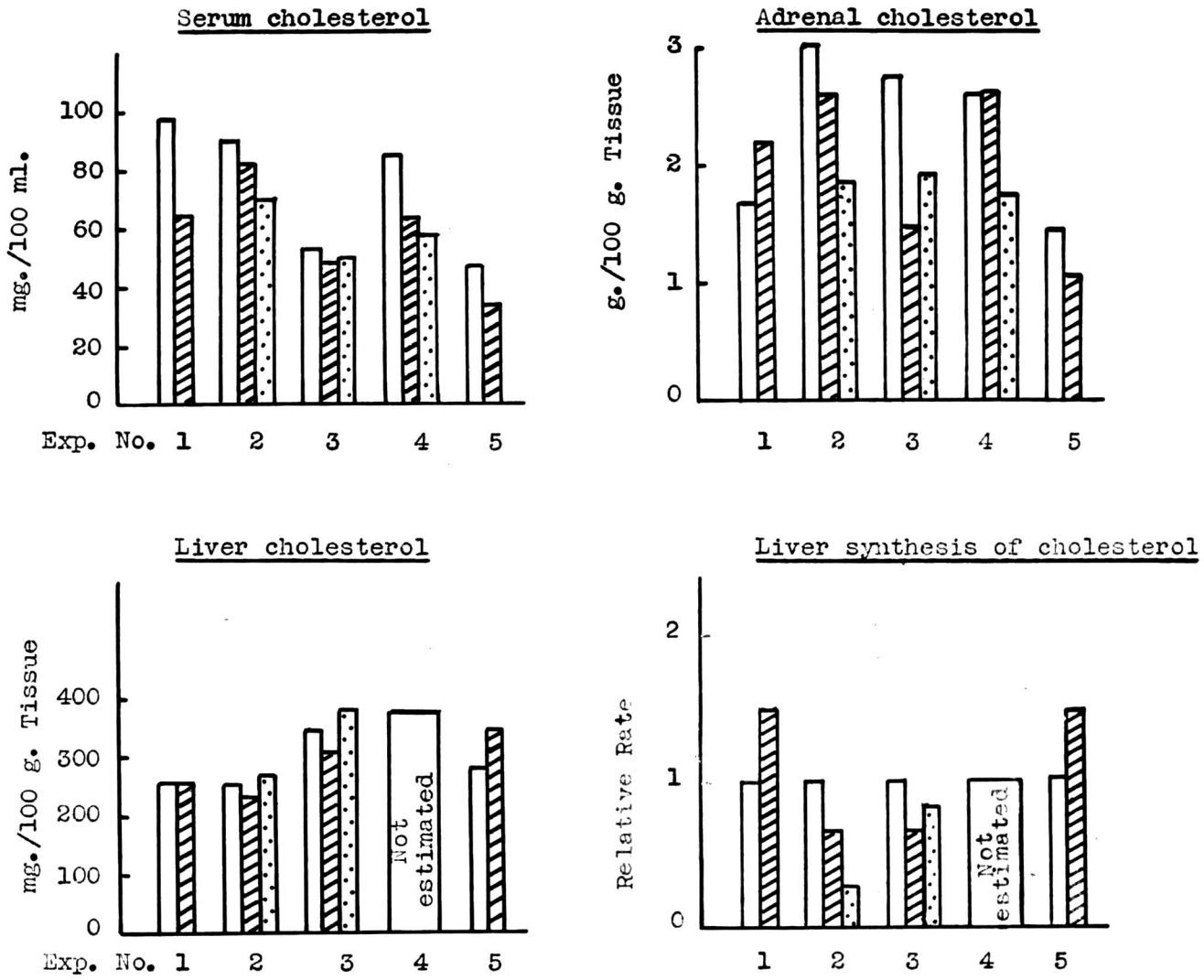
FIGURE 9.

weight was observed.

In the case of ethinyloestradiol the mean plasma cholesterol level rose from an initial pre-treatment value of 17.5 mg./ 100 ml. to a maximum of 46.9 mg./ 100 ml. This greatly elevated level was associated with a dosage of 112 µg. of ethinyl-oestradiol per kg. of body weight per day. Subsequent doubling of this dose failed to alter the value to any significant extent. The plasma cholesterol remained elevated for about three weeks after cessation of treatment but thereafter fell rapidly, reaching the normal range in a further three weeks.

The response to hexoestrol lagged behind the administration of this substance. No increase in plasma concentration of cholesterol was observed while the rabbits received 180 µg. of hexoestrol per kg. of body weight per day for fifteen days. During the next twenty-five days, when the dose was doubled, the plasma cholesterol rose to 23.3 mg./ 100 ml. from an initial pre-injection level of 14.4 mg./ 100 ml. After cessation of treatment the cholesterol concentration of the plasma continued to rise, reaching a maximum value of 39.9 mg.

THE EFFECTS OF ETHINYL OESTRADIOL ADMINISTRATION ON SOME ASPECTS OF CHOLESTEROL METABOLISM IN THE MALE RAT



Key to histograms

Each histogram represents the mean of values obtained from 3 to 6 young male rats, at the termination of the experiment. Ethinyl oestradiol was administered in propylene glycol by subcutaneous injection in every case except in Exp. No. 5. Control rats received propylene glycol only.

□, Controls ▨, Lower dosage ⊡, Higher dosage.

Exp. No.	Dosage (µg./Kg./day)	Duration (days)
1	250	15
2	500 & 1000	13
3	400 & 800	11
4	1300 & 2600	16
5	500 orally	26

FIG. 10

/ 100 ml. fifteen days after the last injection. Thereafter it fell to the normal range within a further fifteen days.

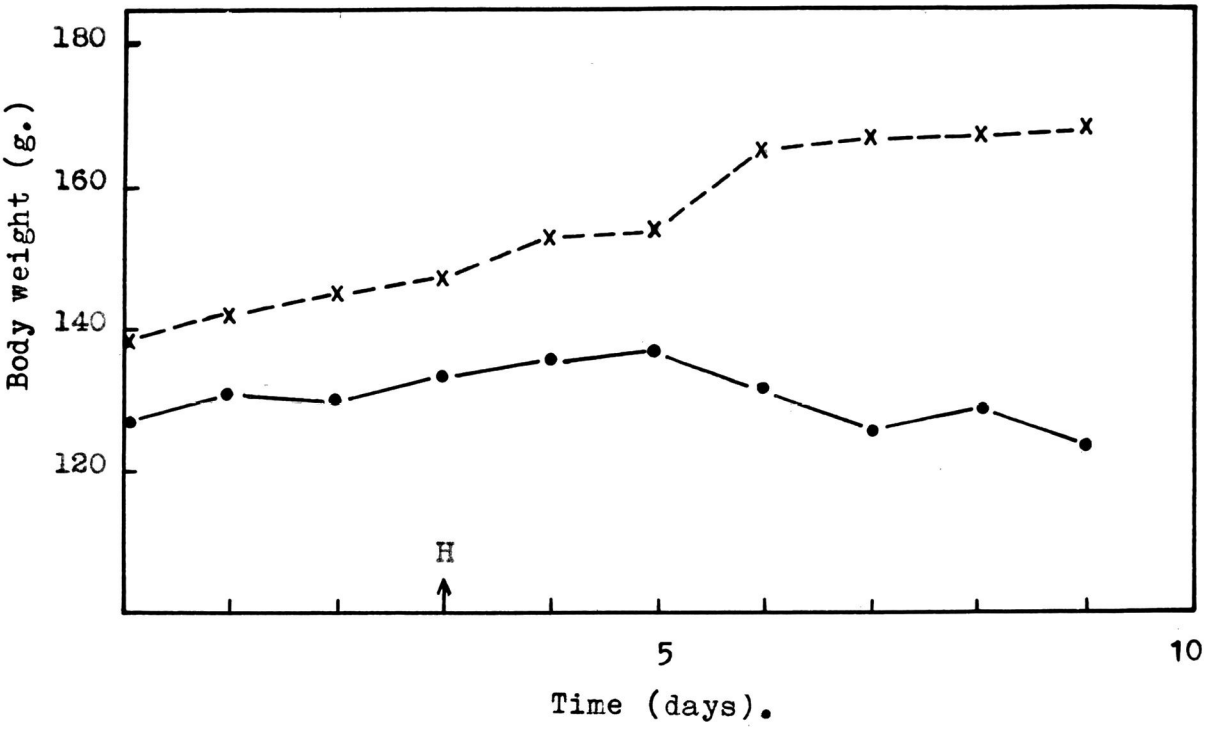
A marked feminizing change was noted in the ethinyloestradiol treated bucks. These animals developed large 'dewlaps' (loose pouches of skin at the throat) which are a secondary sexual characteristic of the female of certain of the heavier breeds of rabbit. Enlargement of the nipples of the breast also occurred. The tendency towards such development in the bucks which received hexoestrol was slight. Incidentally, no permanent loss of fertility was observed in the oestrogen treated animals; one of the bucks which had received ethinyloestradiol fathered a litter a few months after the cessation of treatment.

Rats.

The various oestrogens employed elicited different responses and the results will be presented in separate sections.

Ethinyloestradiol. This oestrogen was used in five experiments at dosage levels in the range 250-2600 μg./ kg./ day for periods of eleven

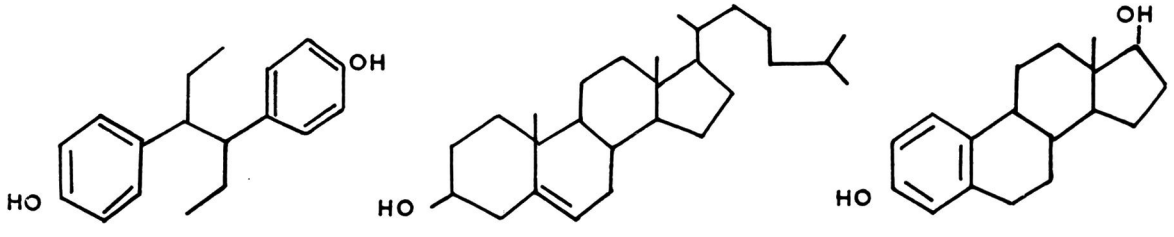
INFLUENCE OF HEXOESTROL ON THE GROWTH OF YOUNG MALE RATS.



Growth curves. H, hexoestrol administration commenced.

•———• , treated rats (4) which received injections of hexoestrol (800µg./kg./ day for 6 days) in propylene glycol.

x- - - - -x , control rats (4) which received injections of propylene glycol.



HEXOESTROL. CHOLESTEROL. OESTRADIOL-17β.

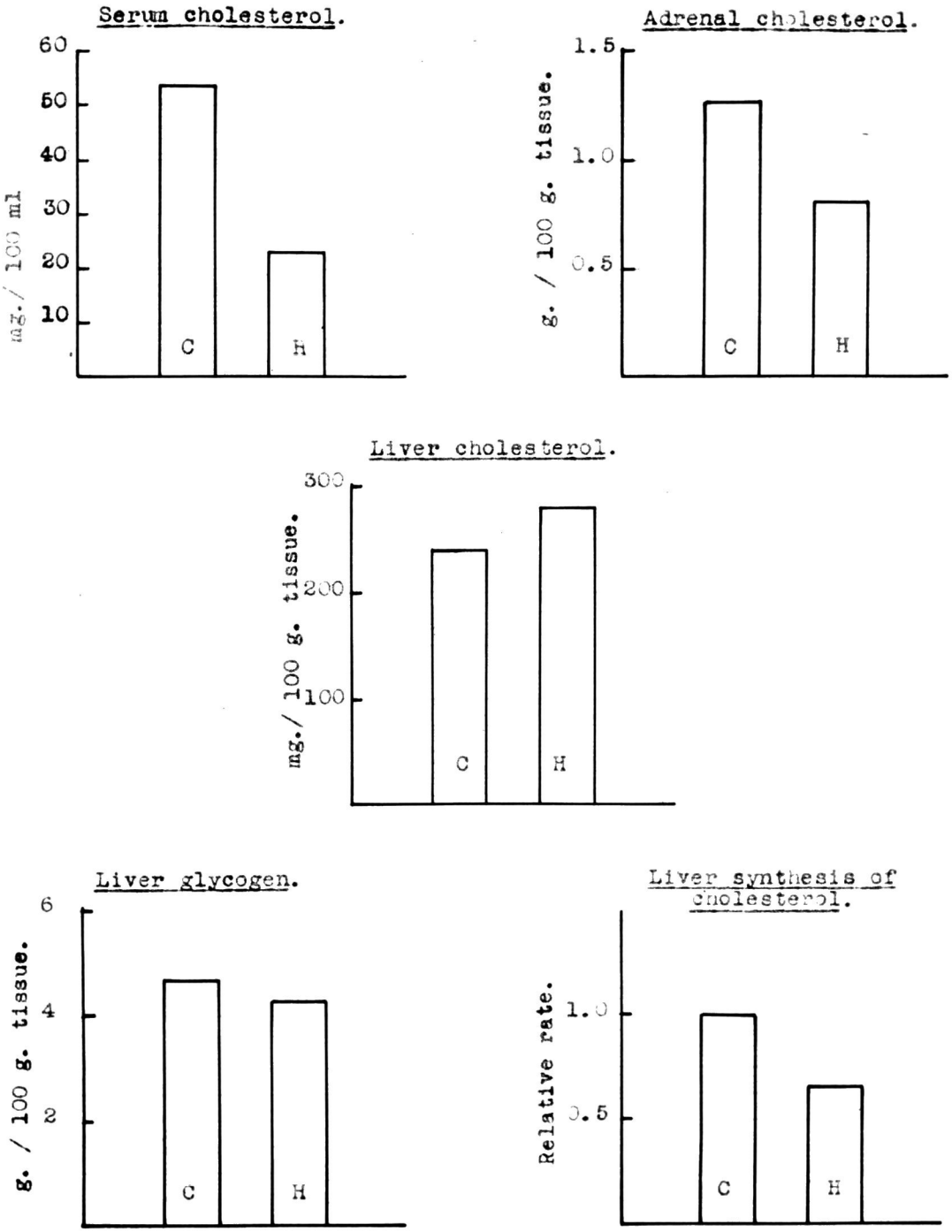
FIGURE 11.

to twenty-six days. Administration of ethinyl-oestradiol always decreased the appetite of the rats and, since pair feeding was not enforced, they gained weight more slowly than did the control animals. The results of this group of experiments have been summarized in Figure 10. There was a tendency towards reduction in the concentrations of both serum and adrenal cholesterol but in neither case was the trend consistent or very marked. Liver cholesterol concentration was unchanged and the rate of hepatic synthesis of cholesterol from acetate *in vitro* was inconsistently affected.

Hexoestrol. This oestrogen was administered to intact rats in two experiments. In both cases the hexoestrol treated rats lost weight markedly while control rats grew normally (Fig. 11). This was possibly due entirely to the anorexia which accompanied the administration of the oestrogen.

In the first experiment the rats received 800 μg. of hexoestrol per kg. of body weight per day for six days. The results have been summarized in Figure 12. Though it has not been shown in the figure, the weights of the pituitaries and

THE EFFECTS OF HEXOESTROL ADMINISTRATION ON SOME ASPECTS OF CHOLESTEROL METABOLISM IN THE MALE RAT (FIRST EXPERIMENT).



C , control rats (4), each of which received 0.1 ml. propylene glycol subcutaneously per day for six days.
H , hexoestrol treated rats (4), each of which received 800 /µg./kg./ day hexoestrol in 0.1 ml. propylene glycol for six days.

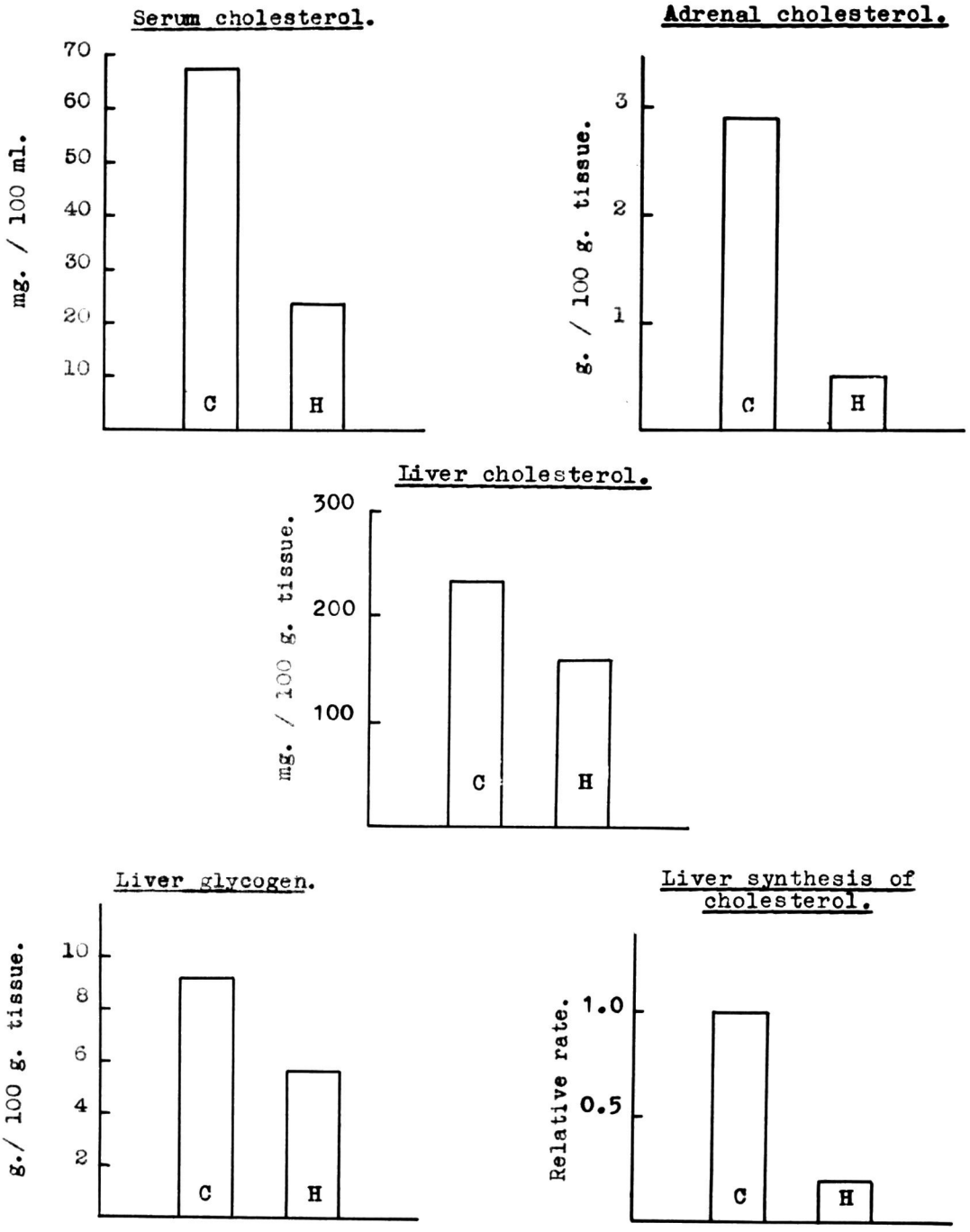
Each column represents the mean value measured at the termination of the experiment.

FIGURE 12.

adrenals of the hexoestrol group did not differ significantly from those of the control group at the end of the experiment. In the hexoestrol treated rats the serum cholesterol and the adrenal cholesterol concentrations were much reduced while the liver cholesterol concentration was unaltered. Nevertheless, since the livers of the oestrogen injected rats weighed less than the livers of control animals, there was a reduction in total liver cholesterol in the treated rats. Liver glycogen was normal but the rate of hepatic synthesis of cholesterol from acetate in vitro was considerably less than the rate observed when slices of liver from control rats were used.

In the second experiment the rats received 850 µg. of hexoestrol per kg. of body weight per day for fifteen days. The results have been summarized in Figure 13. Though it has not been shown in the figure the pituitary and adrenal weights of the treated animals were found to be much higher than those of the control rats at the end of the experiment. While the serum cholesterol concentration of the hexoestrol treated rats fell markedly during the experiment, the haematocrit

THE EFFECTS OF HEXOESTROL ON SOME ASPECTS OF CHOLESTEROL METABOLISM IN THE MALE RAT (SECOND EXPERIMENT).



C , control rats (4), each of which received 0.1 ml. propylene glycol subcutaneously per day for fifteen days.
H , hexoestrol treated rats (4), each of which received 850 µg./kg./ day hexoestrol in 0.1 ml. propylene glycol subcutaneously for fifteen days.

Each column represents the mean value measured at the termination of the experiment, with the exception of serum cholesterol, which was measured on the eleventh day.

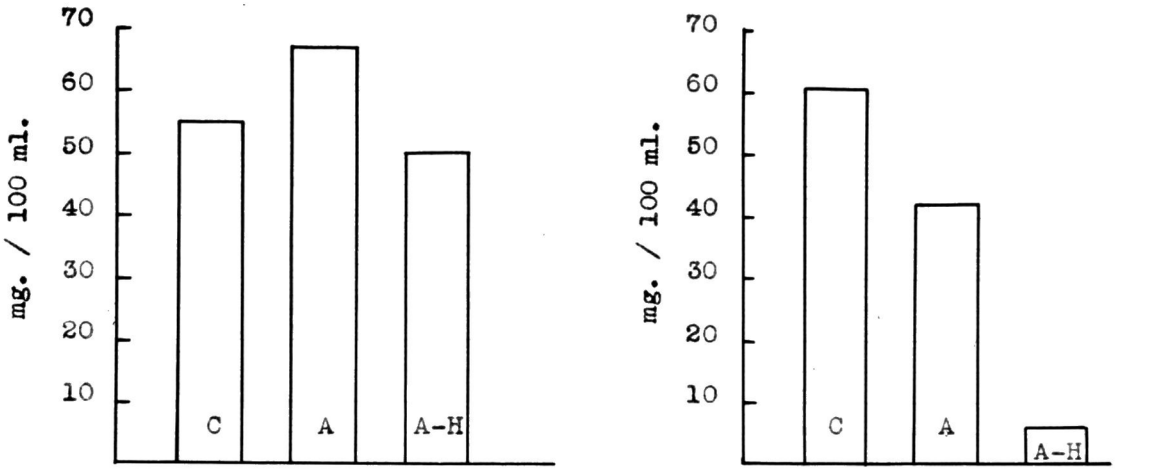
FIGURE 13.

remained at the control value, indicating that the fall in cholesterol level was not attributable to haemodilution. At the termination of the experiment the adrenal cholesterol concentration was found to be less than twenty per cent of that of the control rats and the liver cholesterol was also considerably depleted. (These reductions were not merely changes in concentration; the absolute weights of cholesterol in the tissues were also abnormally low.) Both liver glycogen concentration and the rate of hepatic synthesis of cholesterol from acetate in vitro proved to be much lower than the control values.

Hexoestrol was administered to adrenalectomized rats in two experiments. In the first of these the treated rats received 540 µg. of hexoestrol per kg. of body weight per day for six days. No pair feeding was attempted and consequently both the intact untreated control rats and the adrenalectomized control rats gained weight while the adrenalectomized hexoestrol-treated rats lost weight. The changes in serum cholesterol concentration of these three groups have been depicted in Figure 14. The only group to experience a

THE EFFECT OF HEXOESTROL ON THE SERUM CHOLESTEROL CONCENTRATION OF THE ADRENALECTOMIZED MALE RAT.

FIRST EXPERIMENT: AD LIB. FEEDING.



Serum cholesterol while intact and untreated.

Serum cholesterol at termination of experiment.

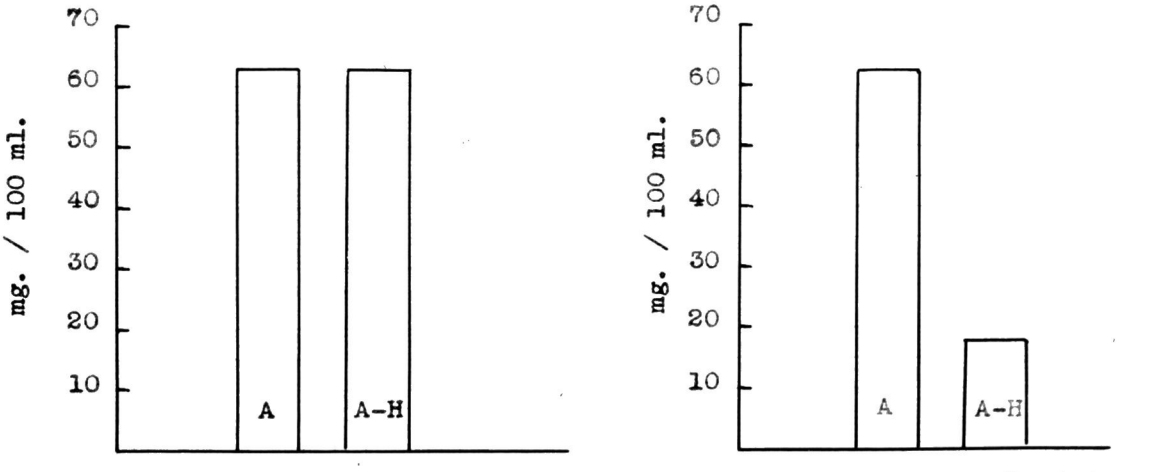
C , untreated control rats (3).

A , adrenalectomized rats (2)

A-H , adrenalectomized rats (3) which received 540 µg./kg./ day hexoestrol for six days.

FIGURE 14.

SECOND EXPERIMENT: GROUP PAIR FEEDING.



Serum cholesterol while intact and untreated.

Serum cholesterol at termination of experiment.

A , adrenalectomized rats (5).

A-H , adrenalectomized rats (6) which received 400 µg./ kg./ day hexoestrol for six days.

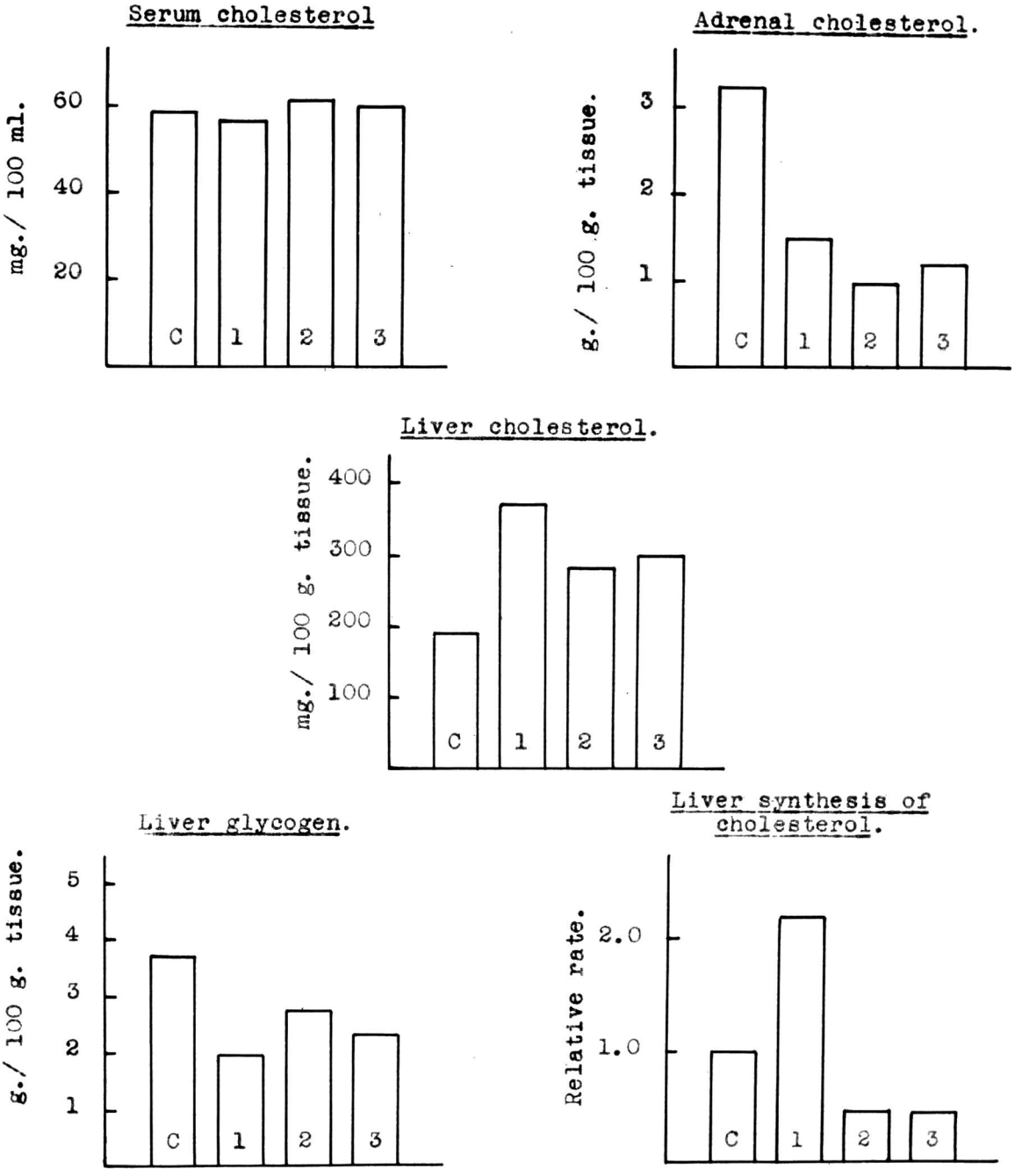
FIGURE 15.

marked decrease in concentration was the adrenalectomized hexoestrol-treated group, in which the serum cholesterol fell from an initial pre-treatment value of 50.2 mg./ 100 ml. to the exceptionally low level of 6.12 mg./ 100 ml. That of the adrenalectomized control rats fell from an initial pre-treatment level of 66.9 mg./ 100 ml. to 42.3 mg./ 100 ml. Haematocrit values were comparable in all groups.

In the second experiment on adrenalectomized rats the treated animals were given 400 µg. of hexoestrol per kg. of body weight per day for six days. Group-pair feeding was enforced and consequently both groups lost weight as the anorexia of the oestrogen treated rats developed. However, the loss of weight in the latter group was more pronounced. The serum cholesterol concentration remained unchanged in the untreated adrenalectomized group at a value of about 63 mg./ 100 ml., while in the adrenalectomized hexoestrol-treated group it fell from 62.6 to 17.7 mg./ 100 ml. No change in haematocrit value was observed. (Figure 15.)

Naturally Occurring Oestrogens. In this experiment oestrone, oestradiol and oestriol were

THE EFFECTS OF NATURAL OESTROGENS ON SOME ASPECTS OF CHOLESTEROL METABOLISM IN THE MALE RAT.



SYMBOL.	TREATMENT.	DOSAGE.
C	Propylene glycol.	0.1 ml./ rat / day.
1	Oestrone.	3.3 - 6.4 mg./ kg./ day.
2	Oestradiol.	1.9 - 3.6 mg./ kg./ day.
3	Oestriol.	4.7 - 9.1 mg./kg./ day

With the exception of the serum cholesterol columns which represent mean values obtained from groups of four rats, each column represents the mean value obtained from two rats which had received the subcutaneous injections detailed above.

FIGURE 16.

administered in relatively large doses to small groups of rats. The appetite of the oestrogen treated rats was reduced and they failed to gain weight. Since group-pair feeding was enforced, the control group gained weight only very slowly. The results of this experiment have been summarized in Figure 16. Though it has not been shown in the figure, the adrenals of the oestrogen treated rats were considerably heavier than those of the control animals. The treated rats maintained normal serum cholesterol levels and haematocrit values throughout the experiment. At the time of sacrifice both the concentration and the total content of cholesterol in the adrenal glands of the oestrogen treated groups were found to be low while, conversely, the liver cholesterol concentration was elevated above the control value.

Unfortunately the liver weights were not recorded in this experiment and consequently it is not known whether there was an absolute increase in liver cholesterol. Liver glycogen concentration was lower in the treated groups than in the control group. The rate of hepatic synthesis of cholesterol from acetate <u>in vitro</u> was approximately half

the control value in the case of the oestradiol treated rats and a similar figure was obtained for the rats which received oestriol. The liver slices from the rats which received injections of oestrone synthesized cholesterol twice as rapidly as did slices from the control animals.

The figures for serum cholesterol were obtained in each case from groups consisting of four rats each and are unequivocal. The other values mentioned were derived from groups of only two rats each and consequently can only be regarded as suggestive of the state of cholesterol metabolism in these animals. This applies particularly to the measurement of the rate of hepatic synthesis of cholesterol in which the spread of values obtained was usually considerable.

DISCUSSION.

Probably the most striking result of this study was the finding that there appears to be a species difference between the rabbit and the rat with respect to the influence of oestrogens on the

level of circulating cholesterol. Since only the plasma cholesterol concentration was studied in the rabbit while several variables were studied in the rat, the following discussion will deal largely with the latter species.

However, it is of interest that the increase in the level of circulating cholesterol which in this work accompanied administration of oestrogens to rabbits met with both confirmation and contradiction in the findings of others. In 1928 Mori and Reiss reported an increase in the blood cholesterol of rabbits following treatment with "Ovarialhormone" which can probably be assumed to be a rather crude mixture of oestrogens. Bruger, Wright and Wiland (1943) administered oestradiol dipropionate to castrate female rabbits and though they concluded that this oestrogen had no influence on blood cholesterol content their data reveal a considerable increase in this value. On the other hand a very striking decrease in serum cholesterol was obtained in female rabbits by Pekkarinen et al. (1952) who administered a massive dose of diethylstilboestrol (about 4.3 mg./ kg. of body weight over a period of about twenty-four hours) in an acute

experiment. Similarly Lewis, Masson and Page (1953) observed that the administration of 700 μg. of diethylstilboestrol per kg. of body weight per day for thirteen days to castrated rabbits of both sexes resulted in a decrease in the level of serum cholesterol to the pre-castration level. Since the level had not risen to any great extent after castration, the return to normalcy did not constitute a very pronounced decrease. The contradiction inherent in these latter studies of the results presented here, may be more apparent than real since the conditions and procedures were so different in the seemingly conflicting experiments.

It has been claimed that oestrogens depress the activity of the thyroid gland in the rabbit (Brown-Grant, 1955) but can have a stimulating effect in the rat (Soliman and Reineke, 1955), and the association of hypercholesterolaemia with hypothyroidism and of hypocholesterolaemia with hyperthyroidism is well known (Boyd and Connell, 1936; Rosenman, Byers and Friedman, 1952b). Thus the apparent species difference alluded to at the beginning of this discussion may derive from a difference in response of the thyroid glands of the

two species to oestrogens.

Turning now to the experiments performed on male rats the generalisation might be made that the oestrogens employed in these studies differed only quantitatively in their influence on cholesterol metabolism. Nevertheless, only hexoestrol produced significant effects in this respect and consequently it is proposed to limit this discussion to a consideration of the effects of this oestrogen only.

It has been shown that hexoestrol has a profound effect on cholesterol metabolism in the male rat and this is in general agreement with the findings of other workers using different oestrogens. In depressing the level of serum cholesterol and adrenal cholesterol, hexoestrol has been found to behave in a similar manner to diethylstilboestrol (Levin, 1945; Pekkarinen et al., 1952). The inhibitory effect of hexoestrol on hepatic synthesis of cholesterol from acetate is in accord with the finding by Rosenman, Friedman and Byers (1952) that injections of oestradiol benzoate reduced the rate of hepatic synthesis of the sterol in the rat. These workers drew this conclusion from the de-

creased biliary cholesterol concentration which they observed in the treated animals. Such a decrease had previously been shown to be indicative of a subnormal rate of hepatic cholesterologenesis. (Byers and Friedman, 1952).

The administration of oestrogens to rats is accompanied by a loss of body weight and, in the case of synthetic oestrogens, this might be due entirely to decreased food consumption consequent to loss of appetite (Meites, 1949). It is known that the livers of fasted rats largely lose their ability to synthesize cholesterol from acetate (Tomkins and Chaikoff, 1952) and the possibility exists that hepatic cholesterol synthesis depends to some extent at least on supplies of available glycogen (Haugaard and Stadie, 1952). With these points in mind, it might be argued that the much decreased rate of hepatic synthesis of cholesterol from acetate in the rats receiving hexoestrol was due merely to decreased stores of liver glycogen. The normal liver glycogen figures and the decreased rate of hepatic cholesterol synthesis observed in the first experiment on intact animals would appear to rule out this possibility.

The reduced rate of hepatic synthesis of

cholesterol in the hexoestrol treated rats may have contributed to the considerable drop in serum cholesterol observed in these animals since most of the circulating cholesterol is synthesized in the liver (Gould et al., 1951). Whether this is the only way in which hexoestrol reduces the serum cholesterol level is not known at present. It is noteworthy that Levin (1945), using diethylstilboestrol and adult male rats, found that the hypocholesterolaemia resulting from administration of the oestrogen for a certain period of time, was much greater than that observed in untreated rats starved completely for the same period, and that the body weight loss in the starved animals exceeded that of the oestrogen treated rats. Thus it would seem that the decreased level of serum cholesterol observed in the hexoestrol treated rats in the present study was not due to inanition.

It is of interest that a substantial decrease in both serum and adrenal cholesterol was observed after hexoestrol administration in the shorter experiment, while no hypertrophy of the pituitary or of the adrenals was observed. Similarly liver cholesterol and glycogen levels were as yet un-

affected. More prolonged administration of the oestrogen produced changes in the pituitary and adrenal glands which are in agreement with the findings of other workers using both hexoestrol and diethylstilboestrol (Burkl, Kellner and Lindner, 1954; Lindner, Satke and Voelkel, 1950; Vogt, 1945; Teague, 1942). This treatment also markedly reduced liver cholesterol and liver glycogen. Other workers have observed a lipid depleting action of oestrogens on the liver (Shipley et al., 1950; Teague, 1942) but the literature seems to contain few references to a glycogen depleting effect. On the contrary most workers have reported accumulation of glycogen in the livers of rats receiving oestrogens (Griffiths et al., 1941; Janes and Nelson, 1940; Long, 1942; Teague, 1942). Nevertheless the experimental conditions prevailing in this work were different from those in most of the studies in which an accumulation of glycogen was observed. In the present investigation there was no period of enforced fasting before sacrifice since this would have rendered impracticable our comparative measurements of the rate of hepatic synthesis of cholesterol from acetate (Tomkins and

Chaikoff, 1952). In the opinion of Walaas (1952) a marked increase in liver glycogen during oestrogen administration only results if the animals are fasted before examination. It is possible that in the present work the very low level of liver glycogen observed in the rats which received hexoestrol for fifteen days is explained by the prolonged decreased food consumption of the animals.

It has been shown that the hypocholesterolaemic response to hexoestrol in the male rat is not dependent upon the presence of adrenal glands. If hexoestrol was behaving in the same manner as other oestrogens which have been observed to possess a lipotropic action this finding would seem to contradict the notion that they might exert this action via the pituitary-adrenal axis, as was tentatively suggested by Levin (1945) with respect to diethylstilboestrol. Nevertheless oestrogens have been shown to induce hypertrophy of the pituitary in rats and apparently stimulate the elaboratory and secretory functions of the gland (Wolfe, 1949; Burkl et al., 1954).

The structural relationship of hexoestrol

to cholesterol (Fig. 11) suggests that the former might have interfered with the synthesis of the latter by blocking certain enzyme systems on the biosynthetic pathway to the sterol. This suggestion has found no support in preliminary studies in which hexoestrol has been added to normal liver slices actively synthesizing cholesterol from acetate in vitro (Boyd and McGuire, unpublished observation).

Hexoestrol might conceivably have damaged the livers of the treated rats to an extent which impaired their synthetic powers. However, histological examination of the liver tissue revealed no abnormalities.

So far the results of the experiments have largely been discussed as though they were attributable to a direct action of oestrogens on cholesterol metabolism. However, it is very probable that oestrogen administration gave rise to an endocrine imbalance in the treated animals and the disturbances most likely to influence cholesterol metabolism would be those involving changes in the level or effectiveness of thyroid hormone or insulin.

The reports in the literature on the influence of oestrogens on the thyroid gland in the rat are conflicting. In 1955 Soliman and Reineke reported that oestradiol benzoate increased the uptake of radio iodine by the rat thyroid *in vivo* and in 1956 Feldman obtained a similar effect using oestrone. On the other hand, several years previously it had been claimed that oestrogens decreased the uptake of I^{131} by the rat thyroid *in vivo*, while dietary changes could reverse the effect or alter the response. (Money, Kraintz, Fager, Kirschner and Rawson, 1951). Lindner *et al.* (1950) found that the thyroid glands of female rats which were carrying implanted hexoestrol pellets were histologically dormant, while Burkl *et al.* (1954) observed that, under similar conditions, the thyroid glands at first increased markedly in weight and activity but soon returned to normal weight and quickly lost their signs of increased activity. It would seem that low doses of oestrogen can stimulate the thyroid while large doses can have an inhibitory effect (Noach, 1955).

The hypothyroid state in rats is associated with a decreased rate of hepatic cholesterol syn-

thesis and hypercholesterolaemia, while hyperthyroidism is associated with an increased rate of hepatic cholesterol synthesis and hypocholesterolaemia (Rosenman, Byers and Friedman, 1952b; Marx, Gustin and Levi, 1953; Dayton, Dayton and Kendall, 1954). However it seems unlikely that the observed effects are attributable simply to a deranged thyroid state since the animals receiving hexoestrol exhibited simultaneously a decreased rate of hepatic synthesis and hypocholesterolaemia. Post mortem examination of the thyroid tissue of these rats gave no indication of increased activity.

The high plasma cholesterol concentration associated with diabetes mellitus and the return to normal concentration with successful insulin therapy in humans is well known (Rabinowitch, 1929). Similarly Cagan, Sobel, Nichols and Loewe (1954) found the serum cholesterol level of the alloxan-diabetic rat to be elevated considerably above normal. Hotta and Chaikoff (1952) reported that liver slices from alloxan-diabetic rats exhibited a rate of synthesis of cholesterol from acetate in vitro which was above normal and, further, that treatment of these rats with insulin reduced this in vitro

synthesis rate. Thus insulin appears to favour reduction both of serum cholesterol concentration and of hepatic synthesis of cholesterol. Both of these values were markedly lowered in the hexoestrol treated rat. Stilboestrol, oestrone and oestradiol propionate have all been observed to increase pancreatic insulin in rats (Griffiths and Young, 1940; Fraenkel-Conrat, Herring, Simpson and Evans, 1941) and Vogt's studies (1955) revealed that hexoestrol induces a state of hypocorticoidism in this species. Thus oestrogens have been shown to produce conditions which would probably enhance the action of insulin, and it might be suggested that this explains some of the observed effects of hexoestrol on cholesterol metabolism. However, the low liver glycogen levels detected in one of the experiments casts considerable doubt on the merit of this suggestion. Pancreatic tissue taken from the hexoestrol treated rats presented a normal histological picture.

In the experiments described here it was not possible to measure the rate of degradation of cholesterol or the total carcase cholesterol of the oestrogen treated rats. Knowledge of these

aspects of the problem would contribute considerably to our understanding of the mechanisms involved in the production of the observed effects.

In conclusion it may be said that the diversity of metabolic effects of a substance such as hexoestrol makes the existence of a single isolated mechanism unlikely. It is far more probable that the abnormalities observed in this study represented the resultant of several types of metabolic disturbance.

SUMMARY.

(i) The effects of administering synthetic oestrogens (ethinyloestradiol and hexoestrol) and natural oestrogens (oestrone, oestradiol and oestriol) on some aspects of cholesterol metabolism in the male rat and the male rabbit have been studied.

(ii) In the rat, only hexoestrol had a pronounced effect on cholesterol metabolism. Administration of this oestrogen was accompanied by striking decreases in both serum and adrenal cholesterol

concentrations and in the rate of synthesis of cholesterol from acetate by liver slices *in vitro*. It was shown that the hypocholesterolaemic effect of hexoestrol was also demonstrable in the absence of the adrenal glands.

(iii) In contrast, both hexoestrol and ethinyloestradiol produced a marked hypercholesterolaemia in the male rabbit. No other parameters of cholesterol metabolism were investigated in this species.

(iv) Mechanisms possibly involved in the production of the observed effects have been suggested and discussed.

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APPENDIX.

FURTHER INVESTIGATIONS SUGGESTED BY THIS WORK.

It was not possible in the foregoing studies to determine the rate of degradation of cholesterol in any of the experimental animals, and the rate of hepatic synthesis of the sterol could only be determined in some cases. It would seem desirable to study these processes in most investigations on cholesterol metabolism. The value of the *in vitro* method of measuring hepatic synthesis rate described here would be considerably enhanced by concurrent bile acid analysis of bile itself and/or of faeces. Estimation of the radio-activity of the saponifiable fractions of these latter after the administration of cholesterol, labelled in the nucleus with ^{14}C, would seem to constitute a simple method of guaging the rate of *in vivo* cholesterol degradation.

Turning to the suggestion of specific problems, a study of the effects of progesterone administration on the level of plasma cholesterol in the intact and ovariectomized rabbit might help to

explain the hypocholesterolaemia of pregnancy observed in this species.

In an investigation mentioned in the foregoing but not described in full, the author observed that liver slices prepared from adrenalectomized-castrated male rats synthesized cholesterol from acetate *in vitro* much more rapidly (ca. five times) than did slices from intact control rats. Removal of either the adrenals alone or the testes alone did not produce this effect. Since this was a very small-scale study it requires to be repeated and investigated further.

With regard to the experiments designed to allow the study of the effects of oestrogens on cholesterol metabolism, it might be rewarding to continue attempts to find conditions in which the *in vitro* effects of hexoestrol (etc.) on cholesterol biosynthesis could be readily studied.

In view of the apparent species difference which exists between rats and rabbits with respect to changes in the level of circulating cholesterol brought about by oestrogen administration, it would probably be informative to measure the thyroid activity of the treated animals. This might be done

in several ways but the most commendable is probably by the measurement of serum protein-bound iodine.

The author has suggested that the effects of hexoestrol on cholesterol metabolism in the male rat might be due to a hormonal imbalance induced by the oestrogen. A study of the effects of administering the oestrogen to hypophysectomized rats, to thyroidectomized rats, to pancreatectomized rats and to castrated rats should enable attention to be focussed on one or more of the endocrine glands.

Lastly, the author's suggestion that hexoestrol may influence cholesterol metabolism by inducing an increased secretion of insulin might be followed up quite simply by performing a glucose tolerance test on the oestrogen treated animals. The results of such an experiment would not, of course, constitute conclusive evidence for or against the suggested mechanism.

EXPERIMENTAL PROCEDURES.

1. Routine Collection of Blood from Rats and Rabbits.

(a) Rats. Blood was collected from the tail in the following manner. The tail was warmed for a minute or two in water and examined for suitable points at which to cut. These points showed up as pale pink bands which coincided with slight indentations in the skin of the tail when the latter was pulled gently away from the rat. They were of course the sections of tissue which lay between the caudal vertebrae. While still warm the tail was placed on a cork block and a strong sharp surgical blade (Gillete, Shape 'A') was passed quickly through the tail so that a section of it was removed.

The blood (ca. 0.5 ml.) was collected in a 1 x ¼" glass tube, the tail being 'milked' by gentle stroking if required to perpetuate the flow of blood. The corked tube was allowed to stand at 37° C. for 30 mins. by which time the clot had retracted somewhat. The uncorked tube was then placed in a

15 ml. centrifuge tube and the latter was spun at ca. 1000 r.p.m. for 10 mins. by the end of which time the serum was easily pipetted off.

When sufficient blood had been collected a tourniquet of wet cotton thread was applied to the tail just above the wound and the rat was returned to its cage. Usually the animal had removed the cotton, which did not require to be tightly tied, within twenty-four hours. The rats showed no signs of discomfort after the treatment and no cases of infection of the wounds were observed. The operation appeared to be painless in young rats but caused older rats varying degrees of momentary distress. By this method blood could be obtained repeatedly.

(b) Rabbits. Blood was collected from the marginal ear vein in the following manner. The hair was clipped short around the edge of the ear and the latter was warmed in water for a few minutes. The ear was then dried rapidly, wiped with a benzene soaked swab, which appeared to cause vaso-dilation, and re-dried. A very small incision was made in the vein by means of a fine, acutely

pointed, surgical blade (Swann Morton, No. 11) and the blood (1.5 to 3 ml.) was collected in an oxalated 15 ml. centrifuge tube. Centrifugation at ca. 2,500 r.p.m. for 15 mins. yielded 0.7 to 1.5 ml. of plasma.

The size of the incision enabled haemostasis to be easily and rapidly achieved by the application of slight pressure. By this method blood could be collected every few days for at least three months without apparent damage to the ear.

2. Preparation of Lipid Extracts from Fluids and Tissues.

(a) Blood Plasma or Serum. At least five volumes of a mixture of acetone and ethanol (1: 1, v / v) were added with shaking to a known volume of plasma or serum in a graduated tube fitted with a ground glass stopper. The contents of the tube were then refluxed for a few minutes to allow all the cholesterol to pass into the liquid phase and the cooled extract was diluted to a known volume with the acetone-ethanol mixture. After mixing, the precipitated protein was centrifuged down

and aliquots of the clear supernatant could be removed by means of a pipette, the tip of which was covered with a small wrap of cotton wool to trap any particles of solid matter.

In the case of the rat, 0.2 ml. of blood serum was refluxed with ca. 2.5 ml. of solvent and the volume adjusted to a final volume of 7.5 ml., while 0.5 or 1.0 ml. of rabbit blood plasma was refluxed with 2.5 or 5.0 ml. of solvent and diluted to a final volume of 7.5 or 15 ml. In preliminary experiments it had been found that such proportions of solvent to solution ensured virtually complete extraction of the cholesterol.

(b) Liver. The weighed portion of liver (ca. 1 g.) was homogenized with twice its volume of physiological saline in an all glass apparatus and an aliquot (usually 1.0 ml.) was refluxed with acetone-ethanol (usually 15 ml.) for a few minutes in a 25 ml. standard flask. After cooling, diluting to the mark with the solvent and mixing, the suspension was filtered through Whatman paper No. 42, and the clear extract stored in glass stoppered tubes.

(c) Adrenals. The weighed glands (two) were ground up in a glass tube with acetone-ethanol (ca. 5 ml.) which was then refluxed for a few minutes and decanted into a 50 ml. standard flask. The grinding and refluxing with fresh volumes of solvent was carried out a further four times in order to extract the cholesterol from the glands exhaustively. The combined extracts were cooled and diluted to 50 ml. with the solvent.

METHODS OF ANALYSIS AND MEASUREMENT.

1. Cholesterol Concentration.

The method used was that of Schoenheimer and Sperry as modified by Sperry and Webb (1950). This was scaled down to enable the estimation of about 20 µg. of cholesterol to be made accurately, as was sometimes necessary in the studies on male or pregnant female rabbits. The dried digitonide was dissolved in 0.5 ml. of acetic acid and 1.0 ml. of the acetic anhydride-sulphuric acid reagent was added. After colour development the solutions were centrifuged at 2,500 r.p.m. in an 'M.S.E. Minor' centrifuge for one minute to sediment any

insoluble matter. The optical density was measured in a Spekker absorptiometer using Ilford filter No. 607 (maximum transmission 580 mμ . onwards) and glass cells of capacity 1.0 ml. and light path 2 cm.

In order to check the accuracy of the method in determining cholesterol concentrations of the low order encountered in the blood plasma and serum of rodents, the following experiment was performed.

The cholesterol contents of human blood plasma samples were determined by the Sperry and Webb modification of the Schoenheimer-Sperry method and by the micro procedure described above. The results were in good agreement (see Table below).

Several dilutions of these plasmas were then prepared and their cholesterol concentrations were determined by the micro procedure. With one exception, the values so obtained agreed well with the actual concentrations of cholesterol (calculated from a knowledge of the degree of dilution of stock human plasma) over the range encountered in the foregoing investigations (see Table below). The exceptional result referred to would appear to indicate that the accurate estimation of cholesterol

in 0.2 ml. of plasma is not possible by this micro procedure if the cholesterol concentration is as low as 10 mg. / 100 ml. of plasma. Such concentrations were once observed in adrenalectomized hexoestrol-treated rats.

Incidentally, this experiment provided a useful check on the efficiency of the extraction procedures described earlier, since some of the samples were treated in the manner described for rat serum and the remainder in that described for rabbit plasma (Appendix, p. 102).

Actual cholesterol concentration of sample. (mg. / 100 ml.)	Cholesterol concentration determined by micro procedure. (mg. / 100 ml.)	Extraction method employed.	"Recovery" by micro procedure. (%)
226 (Undiluted human plasma.)	213		94.3
195 (Undiluted human plasma.)	198		102
107	107	That used for rat serum (Appendix, p. 102).	100
80.3	84.2		105
60.2	63.4		105
45.2	44.4		98.3
33.9	34.6		102
25.4	26.9		106
12.7	16.9		(133)
71.2	71.2	That used for rabbit plasma (Appendix, p. 102).	100
48.0	51.7		108
24.0	25.0		104
12.0	11.6		96.6

Mean "Recovery" (excluding bracketed result): 102 ± 4.22 %

2. Glycogen (plus Glucose) Concentration.

A method was required for the estimation of glycogen plus glucose in rat liver which would be of sufficient accuracy to allow comparison of different groups of livers.

The method used was essentially that described by van der Vies (1954) with the following modifications:-

(a) The liver homogenate was extracted with trichloracetic acid (T.C.A.) in a boiling water bath for 15 mins. instead of at room temperature, in order to bring all the glycogen into solution. (Kemp and Kits van Heijningen, 1954.)

(b) The alkaline destruction of glucose in liver was omitted and consequently 0.2 ml. of the T.C.A. extract was used directly in the estimation instead of 2 ml. of a 1 in 10 dilution of the extract.

(c) The glucose standards were treated in the same manner as the extracts, as described in (b).

Though modifications (b) and (c) are trivial,

and though it has been established that boiling T.C.A. dissolves all the glycogen present in liver tissue, it was felt that an appreciable error might arise in measuring the small volumes used in the modified technique. Accordingly two experiments were performed each in the following manner.

Several adjacent portions (ca. 200 mg. each) were cut from the liver of a twenty-four-hour-fasted rat.[1] These were weighed on a tortion balance and homogenized with T.C.A.. The glycogen plus glucose (expressed as 'glycogen') in two such portions was estimated by the modified technique . Aliquots of a standard solution of glycogen in T.C.A. were added to the remaining homogenates and each of these mixtures was estimated by the same technique. After correcting the values so obtained for the 'glycogen' content of the liver portions taken, comparison with the known weights of glycogen added was possible. The results have been summa-

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1. Such a liver was used in order that considerable additions of glycogen could be made to it without exceeding the normal range of values met with in these studies. Additionally, any errors involved in estimating the minute quantity of glycogen present in 200 mg. of fasted-rat liver by this method would be too small to disguise any errors of measurement, inherent in the method.

rized in the table on the following page.

It can be seen that with one exception these results show the method to possess a suitable degree of accuracy.

In the experiments described in the foregoing sections only one rat was encountered having a liver glycogen concentration of less than 2.20 g. / 100 g.; i.e. in only one case was the liver glycogen low enough to render its accurate estimation unlikely.

3. Hepatic Synthesis of Cholesterol from Sodium (1-^{14}C) - Acetate in Vitro.

The rate of this synthesis was measured by the method of Boyd (to be published) which is, briefly, as follows.

Slices (0.07 mm. thick) cut by a mechanical tissue chopper (McIlwain and Buddle, 1953) from the livers of control and treated rats were incubated for 3 hr. at 37°C. under 100 % oxygen in Krebs phosphate solution (Krebs and Henseleit, 1932) in the presence of 3 μ.M. of sodium (1 - ^{14}C) - acetate. The slices were saponified with boiling alcoholic potassium hydroxide and the alkaline

"Glycogen" content of fresh liver. (g. / 100 g.)	Actual glycogen addition to liver. (A.) (g. / 100 g.)	Estimated glycogen addition to liver. (B.) (g. / 100 g.)	Recovery. $(\frac{B}{A} \times 100)$ (%)
0.248	0.475	0.462	97.4
0.248	1.05	0.942	90.3
0.248	1.44	1.33	92.0
0.410	1.49	1.54	103
0.248	2.47	2.54	103
0.410	2.89	2.72	94.2
0.410	3.67	3.72	101
0.248	3.79	4.05	107
0.410	5.35	5.47	102
0.410	6.48	6.46	99.9
		Mean recovery:	99.0 ± 5.41 %

solution was then extracted with petroleum ether. After drying over sodium sulphate this extract was evaporated to dryness and the residue dissolved in a few ml. of ethanol-acetone solution (1: 1, v/v). An excess of an aqueous alcoholic solution of digitonin was added to the acidified solution of the residue and, after standing overnight, the cholesterol digitonide was separated by centrifugation and dried. A tared portion of the digitonide was combusted to carbon dioxide which was converted to barium carbonate. The latter was plated in an "infinitely thick" layer and the radio-activity of the tared sample was determined by means of a thin end-window Geiger-counter, employing a "Panax" power unit and scaler. Comparison of the radio-activity of the barium carbonate samples obtained from livers of control and treated rats enabled the relative rate of hepatic synthesis of cholesterol from acetate to be calculated.

PURIFICATION OF SOLVENTS USED IN ESTIMATION OF CHOLESTEROL.

Acetic Acid. The "A.R." grade reagent was refluxed for several hours with finely ground potassium dichromate (30 g./ 1. of acetic acid) and then distilled.

Acetic Anhydride. The "A.R." grade reagent was distilled.

Acetone. The commercial grade reagent was refluxed for several hours with potassium permanganate (40 g./ 1. of acetone) and then distilled.

Ethanol. The commercial grade was refluxed for eight hours with sodium hydroxide pellets (20 g./ 1. of ethanol) and then distilled onto quick lime and left overnight. After filtration the ethanol was redistilled.

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PREGNANCY STUDIES.

TABLE I. SERUM CHOLESTEROL VALUES OF FOUR RATS ALL OF WHICH LITTERED ON THE SAME DAY.

The boxed in values are those determined immediately before parturition. A graph has been constructed from the mean values. (Figure 1, facing p. 32.)

Date.	31-8	14-9	21-9	28-9	5-10	12-10	19-10	26-10	9-11	17-11
Rat No.	Serum cholesterol concentration (mg./ 100 ml.)									
R.1.3.	83.9	72.3	53.8	98.5	52.1	50.7	59.6	76.8	73.1	63.3
L.1.3.	69.3	67.6	43.0	89.5	44.5	67.1	85.3	83.6	82.8	62.0
L.3.3.	64.7	74.0	47.4	85.8	56.3	76.4	81.8	117	93.4	69.6
L.4.3.	67.0	73.6	52.5	81.7	61.5	80.5	80.6	88.5	84.3	70.7
Mean.	71.2	71.9	49.2	88.9	53.6	68.3	76.8	91.5	84.4	66.4

TABLE Ia. SERUM CHOLESTEROL VALUES OF FOUR UNMATED CONTROL FEMALE RATS.

A graph has been constructed from the mean values. (Figure 1, facing p. 32.)

Date.	7-12	14-12	21-12	28-12	4-1	11-1
Rat No.	Serum cholesterol concentration. (mg./100 ml.)					
R.6.1.	79.2	85.1	90.0	80.6	92.0	100.2
R.6.2.	73.5	68.3	70.4	79.4	77.4	68.8
R.1.2.	79.4	68.3	81.1	91.8	85.6	76.4
R.3.4.	85.1	74.2	85.1	92.5	78.0	82.3
Mean	79.3	74.0	81.7	86.1	83.3	81.9

TABLE 1b. HAEMATOCRIT VALUES FROM FOUR MATED RATS, ALL OF WHICH LITTERED ON THE SAME DAY.

A graph has been prepared from the mean values. (Figure 1, facing p. 32.)

Date.	31-8	14-9	21-9	28-9	5-10	12-10	19-10	26-10	9-11	17-11
Rat No.	Haematocrit Value. (Parts per thousand.)									
R.1.3.	490	470	440	350	400	400	410	485	530	470
L.1.3.	480	455	440	358	400	500	515	470		500
L.3.3.	460	450	390	355	410	430	450	520	550	
L.4.3.	480	480		395	420	445	500	500	520	490
Mean.	478	464	423	365	408	444	469	494	533	487

TABLE 1c. HAEMATOCRIT VALUES OF FOUR UNMATED CONTROL FEMALE RATS.

A graph has been constructed from the mean values. (Figure 1, facing p. 32.)

Date,	7-12	14-12	21-12	28-12	4-1	11-1
Rat No.	Haematocrit value. (Parts per thousand.)					
R.6.1.	424	415	443	463	450	454
R.6.2.	418	418	454	450	450	465
R.1.2.	467	426	450	435	458	439
R.3.4.	481	431	464	445	396	429
Mean.	448	423	453	448	439	447

TABLE 2. SERUM CHOLESTEROL VALUES (EXPRESSED AS PERCENTAGES OF THE PRE-MATING VALUES) OF TWELVE MATED RATS.

The boxed in mean value is that determined just before parturition. A graph has been constructed from the mean values. (Figure 2, facing p. 33.)

Days after mating.	2	3	4	5	9	10	12	16	17	19	22	23	24	26
Rat No.	Serum cholesterol concentrations, expressed as percentages of the pre-mating values.													
R.2.			93.0			73.5			88.5				70.0	
R. 3.1.	91.0				86.5			104			101			
R.4.	111				86.0			94.0			93.0			
R.6.		134				84.5			116			87.5		
R.3.2.		112				117			118				118	
R.1.3.				86.5			64.0			118				62.2
R.3.3.	92.4				84.3			101				77.8		
R.4.3.			88.5											64.2
L.1.				97.5			62.0			129			77.8	
L.2.		91.5				76.3			106					87.0
L.3.				115			73.2			133				91.8
L.4.				110			78.5			122				76.3
Mean.	98.1	113	90.8	102	85.6	87.8	69.4	100	107	126	97	82.7	88.6	

Contd. :-

TABLE 2. (Contd.)

Days after mating.	29	30	31	33	36	37	38	40	47	60	61	69	83
Rat No.	Serum cholesterol concentrations, expressed as percentages of the pre-mating values.												
R.2.			95.0										
R.3.1.	109				92.5								
R.4.	108				109								
R.6.		119				96.5							
R.3.2.			131				116		120	87.5			
R.1.3.				60.5				71.0	91.5		87.3	75.5	87.5
R.3.3.		93.0				73.0							
R.4.3.										131			
L.1.				97.0				123	121		120	89.5	108
L.2.			94.6				90.2						
L.3.				118				126	181		144	108	
L.4.				120				120	132		126	105	
Mean.	109	106	107	99.9	101	84.8	103	110	129	109	119	94.5	97.8

TABLE 3. PLASMA CHOLESTEROL CONCENTRATIONS OF MATED RABBITS IN FIRST GROUP.

The boxed in values are those determined immediately before parturition. A graph has been constructed from the values for rabbit No. 015. (Figure 3, facing p.34.)

Date.	20-11	24-11	27-11	1-12	4-12	8-12	11-12	15-12	22-12	30-12	4-1	9-1	12-1	15-1
Rabbit No.	Plasma cholesterol. (mg. / 100 ml.)													
014	35.4	32.5	39.7	29.4	35.6	32.6	32.8	41.1	36.0	47.2	27.2	46.6	22.2	25.0
015	33.9	55.5	53.1	52.6	64.5	52.7	53.2	41.6	54.7	62.5	40.4	48.6	34.2	52.5
016	45.7	54.7	64.2	53.0	82.1	65.4	59.7	50.5	74.9	71.8	45.0	69.5	59.7	87.4
017	28.2	34.4	47.7	39.0	33.8	42.5	49.9	40.5	64.1	41.0	21.0	36.3	20.9	42.2

Date.	18-1	22-1	26-1	29-1	2-2	5-2	9-2	12-2	16-2	19-2	23-2	26-2	2-3	5-3
Rabbit No.	Plasma cholesterol. (mg. / 100 ml.)													
014	15.8	8.60	[8.30]	32.6	57.3	46.1	42.2	50.1	37.8	40.8	39.0	45.5	51.0	61.9
015	27.3	31.6	17.7	27.5	24.6	13.4	[15.1]	57.7	42.2	54.0	68.0	50.0	52.9	58.3
016	41.6	51.4	34.0	22.0	13.1	[14.3]	Rabbit died.							
017	23.6	40.1	34.3	23.2	22.6	28.6	31.7	41.2	29.7	37.5	34.2	34.1	63.6	69.0

Contd. :-

TABLE 3. (Contd.)

Date.	9-3	12-3	16-3	19-3	23-3	26-3	30-3	6-4	8-4	12-4	20-4	23-4	30-4
Rabbit No.	Plasma cholesterol. (mg. / 100 ml.)												
014	67.5												
015	61.1	58.9	52.3	60.0	53.7								
016													
017	69.9	69.0	56.0	21.8	6.30	10.3	22.1	57.1	56.2	46.0	52.8	70.4	48.8

TABLE 4. PLASMA CHOLESTEROL CONCENTRATIONS OF MATED RABBITS IN THE SECOND GROUP.

The boxed in values are those determined immediately before parturition. A graph has been constructed from the mean values. (Figure 4, facing p. 34.)

Date.	31-8	6-9	20-9	23-9	26-9	30-9	3-10	7-10	10-10	14-10	17-10
Rabbit No.	Plasma cholesterol. (mg./100 ml.)										
9	32.2	27.0	27.0	35.6	23.8	17.6	13.8	11.2	9.96	15.0	46.9
10	31.7	31.3	33.5	32.6	25.5	20.5	17.6	12.3	10.2	15.1	57.0
Mean.	32.0	29.2	30.3	34.1	24.7	19.1	15.7	11.8	10.1	15.1	52.0

Date.	21-10	24-10	28-10	31-10	4-11	7-11	11-11	14-11	21-11	28-11	9-12
Rabbit No.	Plasma cholesterol. (mg./100 ml.)										
9	44.0	36.4	27.6	38.8	45.5	38.4	39.0	41.0	43.7	46.2	40.9
10	47.0	37.6	34.4	36.2	45.5	41.0	45.7	37.9	36.2	34.7	39.4
Mean.	45.5	37.0	31.0	37.5	45.5	39.7	43.4	39.5	40.0	40.5	40.2

TABLE 4a. HAEMATOCRIT VALUES FROM MATED RABBITS IN THE SECOND GROUP.

A graph has been constructed from the mean values. (Figure 4, facing p. 34.)

Date.	31-8	6-9	20-9	23-9	26-9	30-9	3-10	7-10	10-10	14-10	17-10
Rabbit No.	Haematocrit value. (Parts per thousand.)										
9	330	360	330	355	340	340	310	335	325	335	310
10	350	360	370	360	340	356	340		320	340	315
Mean.	340	360	350	358	340	348	325	335	323	338	313

Date.	21-10	24-10	28-10	31-10	4-11	7-11	11-11	14-11	21-11	28-11
Rabbit No.	Haematocrit value. (Parts per thousand.)									
9	330	310	310	340	330	310	320	320	325	315
10	330	340	325	310	335	310	310	320	320	330
Mean.	330	325	318	325	333	310	315	320	323	323

A G I N G S T U D I E S.

TABLE 5. SERUM CHOLESTEROL VALUES OF 'AGING' RATS.

Graphs have been constructed from the mean values for each sex. (Figure 5, facing p. 53.)

Age (Days.)		23	46	78	140	154	336
Rat No.	Sex.	Serum cholesterol. (mg./ 100 ml.)					
R.1.	Male	162	81.3	49.0	64.3	57.1	50.7
R.2.	Male	119	80.5	45.5	63.8	56.7	41.7
R.3.	Male	112	79.4	55.5	72.1	76.6	59.4
R.4.	Male	112	79.6	53.2	48.5	67.3	52.9
Mean.		126	80.2	50.8	62.2	64.4	51.2
L.1.	Female	107	83.7	68.8	74.5	93.6	56.4
L.2.	Female	109	91.1	51.7	61.5	70.0	47.8
L.3.	Female	103	78.6	63.3	84.4	84.6	92.0
L.4.	Female	91	81.2	60.3	62.4	72.3	42.5
Mean.		103	83.7	61.0	70.7	80.1	59.7

TABLE 5a. AGING STUDY: COMPARISON OF RESULTS WITH THOSE OF BARGETON, ET AL. (1954) IN THE MALE RAT.

Graphs have been constructed from the mean values. (Figure 5a, facing p. 56.)

Mean body weight (g.).	Author.	31	109	223	313	341	345	351	352	379	380
	Bargeton et al.		120	223	313	341	345	351	352	379	390
Mean serum cholesterol. (mg./100 ml.)	Author.	126	80.2	50.8	62.2	73.3	88.1	74.7	84.2	51.2	74.3
	Bargeton et al.		81.0	62.0	66.0	68.0	69.0	70.0	70.0	73.0	74.0

CASTRATION STUDIES.

TABLE 6. SERUM CHOLESTEROL CONCENTRATIONS OF CASTRATED MALE RATS AND INTACT MALE CONTROL RATS.

A graph has been prepared from the mean values. (Figure 6, facing p. 63.)

Days after castration.		-6	15	36	63	106
Rat No.		Serum Cholesterol. (mg. / 100 ml.)				
L.1.	Castrate	76.1	84.4	84.5	75.0	77.2
L.2.	Castrate	61.3	67.5	75.1	58.5	55.7
L.3.	Castrate	84.3	81.7	89.5	80.8	71.0
L.4.	Castrate	80.0	97.5	94.4	93.0	80.2
L.5.	Castrate	73.4	82.5	84.5	92.7	81.4
Mean.	Castrates	75.0	82.7	85.6	80.0	73.1
R.1.	Intact	77.0	91.5	87.2	71.1	76.5
R.2.	Intact	65.2	70.8	74.0	59.6	59.8
R.3.	Intact	73.4	93.7	84.9	86.1	77.8
R.4.	Intact	72.1	84.5	78.5	64.2	77.0
R.5.	Intact	79.0	100.0	96.6	92.7	80.6
Mean.	Intact	73.3	88.1	84.2	74.7	74.3

TABLE 6a. HAEMATOCRIT VALUES OF CASTRATED MALE RATS AND INTACT MALE CONTROL RATS.

A graph has been prepared from the mean values. (Figure 6, facing p. 63.)

Days after castration.		-6	15	36	63	106
Rat No.		Haematocrit value. (Parts per thousand.)				
L.1.	Castrate	490	450	470	470	460
L.2.	Castrate	480	455	475	460	450
L.3.	Castrate	540	430	460	415	450
L.4.	Castrate	480	400	490	500	480
L.5.	Castrate	490	460	500	520	470
Mean.	Castrates.	496	439	479	473	462
R.1.	Intact	530	510		540	500
R.2.	Intact	480	490	510	510	
R.3.	Intact	520	465	490	510	470
R.4.	Intact	510	480	515	520	
R.5.	Intact	510		490	520	
Mean.	Intact	510	486	501	520	485

TABLE 7. SERUM CHOLESTEROL CONCENTRATIONS OF CASTRATED FEMALE RATS AND INTACT FEMALE CONTROL RATS.

A graph has been prepared from the mean values. (Figure 7, facing p. 63.)

Days after castration.		-7	14	35	62	105
Rat No.		Serum cholesterol. (mg. / 100 ml.)				
L.1.	Castrate	79.4	94.8	88.0	94.0	81.9
L.2.	Castrate	80.5	95.5	85.9	97.8	83.4
L.3.	Castrate	82.5	102	95.5	93.0	80.6
L.5.	Castrate	84.9	86.8	93.0	92.7	80.6
Mean.	Castrate	81.8	94.8	90.6	94.4	81.6
R.1.	Intact	82.0	86.1	77.3	91.8	72.6
R.2.	Intact	62.6	68.2	81.0	65.5	61.7
R.3.	Intact	83.1	91.4	75.2		65.0
R.4.	Intact	87.3	81.8	92.5	96.0	68.3
R.5.	Intact	77.1	85.8	75.6	88.0	72.9
Mean	Intact	78.4	82.5	80.3	85.3	68.1

TABLE 7a. HAEMATOCRIT VALUES OF CASTRATED FEMALE RATS AND INTACT FEMALE CONTROL RATS.

A graph has been prepared from the mean values. (Figure 7, facing p. 63.)

Days after castration.		-7	14	35	62	105
Rat No.	Haematocrit values. (Parts per thousand.)					
L.1.	Castrate	440	480	460	445	460
L.2.	Castrate	460		490	490	450
L.3.	Castrate	490	440	450	470	
L.5.	Castrate	460	430		490	
Mean.	Castrates	463	453	467	474	455
R.1.	Intact	460	455	470	420	470
R.2.	Intact	470	480	470	480	460
R.3.	Intact	470	450	510		460
R.4.	Intact	460	465	480	470	440
R.5.	Intact	450	420	460	500	450
Mean.	Intact	462	454	478	468	456

OESTROGEN STUDIES.

TABLE 8. MEAN VALUES OF PLASMA CHOLESTEROL OF BUCK RABBITS (6) WHICH RECEIVED ETHINYL OESTRADIOL ORALLY.

A graph has been prepared from these figures. (Figure 8, facing p. 77.)

Ethinyl Oestradiol (µg./ kg./ day.)	0	7	14	28	56	112	224	0
Period of Administration. (Days.)	4	23	21	14	27	29	30	76
Plasma Cholesterol (mg. / 100 ml.)	17.5	22.7	24.5	23.0	27.6	33.7	39.1	36.8
		21.8	23.2	29.4	34.3	39.1	38.1	40.8
		24.1	22.9	25.0	34.8	35.4	35.6	35.6
		21.0	24.0	26.5	28.8	46.9	33.4	38.3
		28.6			23.3	40.6	35.6	28.4
					29.1		34.8	26.3
					31.3		40.2	13.4
					30.4			14.2
								14.8
								16.4

TABLE 8a. MEAN HAEMATOCRIT VALUES OF BUCK RABBITS (6) WHICH RECEIVED ETHINYL OESTRADIOL ORALLY.

A graph has been prepared from these figures. (Figure 8, facing p. 77.)

Ethinyl Oestradiol (µg./ kg./ day.)	0	7	14	28	56	112	224	0
Period of Administration. (Days.)	4	23	21	14	27	29	30	76
Haematocrit Values. (Parts per thousand.)	Not measured.	Not measured.	Not measured.	308	303	308	262	255
				322	294	308	238	261
				296	286	308	249	307
					294	285	234	325
					294	281	257	333
					285		250	343
					294		243	350
					279			366
								342
								350

TABLE 9. PLASMA CHOLESTEROL AND HAEMATOCRIT VALUES OF HEXOESTROL TREATED AND CONTROL BUCK RABBITS.

Four rabbits received hexoestrol subcutaneously and two rabbits which acted as controls received the medium, propylene glycol, only. Graphs have been prepared from these mean values. (Figure 9, facing p.77.)

Duration of Experiment. (Days.)	0	7	14	21	28	35	42	49	56	63	70	77	84	91
Hexoestrol Administered. (µg./kg./day.)	0			180		360			0					
Plasma cholesterol. (m./ 100 ml.)														
Hexoestrol rabbits.	14.4	11.0	11.0	8.44	12.2	15.7	23.1	23.3	32.2	35.1	39.9	31.1	19.6	19.1
Control rabbits.	13.7	16.0	17.2	14.7	16.1	15.6	15.1	15.6	16.3	12.3	27.3	20.0	15.9	15.9
Haematocrit. (Parts per thousand.)														
Hexoestrol rabbits.	366	374	387	348	355	358	324	294	269	296	345	350	353	374
Control rabbits.	385	390	403	400	380	340	398	398	375	380	400	390	400	400

TABLE 10. ADMINISTRATION OF ETHINYL OESTRADIOL TO YOUNG MALE RATS.

Histograms have been prepared from these mean values which were obtained at the termination of the experiment. (Figure 10, facing p. 78.)

Experiment No.	1		2			3		
Group.	Control.	Test	Control	Test A	Test B	Control.	Test A	Test B
No. of Rats.	4	4	3	3	3	3	3	3
Ethinyl oestradiol administered. (μg./ kg./ day.)	0	250	0	500	1000	0	400	800
Duration of experiment. (Days)	15	15	13	13	13	11	11	11
Serum cholesterol. (mg./ 100 ml.)	97.8	64.6	90.4	82.2	69.7	53.3	48.2	49.8
Adrenal cholesterol. (g./ 100 g. tissue.)	1.67	2.20	3.00	2.58	1.86	2.77	1.47	1.92
Liver cholesterol. (mg./ 100 g. tissue.)	256	256	256	237	270	342	309	381
Relative rate of hepatic synthesis of cholesterol from acetate.	1.0	1.5	1.0	0.67	0.28	1.0	0.66	0.82

Contd. :-

TABLE 10. (Contd.)

Experiment No.	4			5	
Group.	Control.	Test A	Test B	Control.	Test.
No. of Rats.	3	3	3	4	4
Ethinyl oestradiol administered. (µg./ kg./ day.)	0	1300	2600	0	500
Duration of experiment. (Days.)	16	16	16	26	26
Serum cholesterol. (mg./ 100 ml.)	84.9	63.3	57.4	47.6	34.0
Adrenal cholesterol. (g./ 100 g. tissue.)	2.59	2.62	1.73	1.45	1.05
Liver cholesterol. (mg./ 100 g. tissue.)	Not estimated.			279	348
Relative rate of hepatic synthesis of cholesterol from acetate.	Not estimated.			1.0	1.5

TABLE 11. INFLUENCE OF HEXOESTROL ON BODY WEIGHT OF YOUNG MALE RATS.

Graphs have been constructed from the mean values. (Figure 11, facing p. 79.)

Duration of experiment. (Days.)		0	1	2	3	4	5	6	7	8	9
Rat No.	Treatment.	Body Weight.									
R.1.	0.1 ml. Propylene Glycol/rat/ day subcutaneously from 3rd to 8th day.	126	130	132	132	132	137	147	152	150	152
R.2.		160	166	168	172	176	177	194	191	196	193
R.3.		127	129	134	139	143	147	154	155	158	153
R.4.		139	144	147	147	155	155	165	169	165	172
Mean.		138	142	145	147	153	154	165	167	167	168
L.1.	800 µg./ kg./ day Hexoestrol subcutaneously from 3rd to 8th day.	141	144	146	151	151	154	143	131	143	134
L.2.		120	120	122	130	133	131	127	124	124	120
L.3.		96	101	100	102	104	107	106	102	101	97
L.4.		147	149	151	153	156	157	154	148	148	145
Mean		127	131	130	134	136	137	132	126	129	124

TABLE 12. THE ADMINISTRATION OF HEXOESTROL TO YOUNG MALE RATS (FIRST EXPERIMENT).

Histograms have been prepared from these mean values which were obtained at the termination of the experiment. (Figure 12, facing p.80.)

Group.	Control rats.	Hexoestrol treated rats.
No. of Rats.	4	4
Treatment.	0.1 ml Propylene Glycol subcutaneously per day for 6 days.	800 µg/kg./day Hexoestrol in 0.1 ml. Propylene Glycol subcutaneously for 6 days.
Body weight gain. (g.)	+31	-2.5
Serum cholesterol. (mg. / 100 ml.)	54.0	23.0
Adrenal cholesterol. (g./ 100 g. tissue.)	1.26	0.82
Liver cholesterol. (mg./ 100 g. tissue.)	242	281
Liver glycogen. (g./ 100 g. tissue.)	4.68	4.27
Relative rate of hepatic synthesis of cholesterol from acetate.	1.0	0.65

TABLE 13. THE ADMINISTRATION OF HEXOESTROL TO YOUNG MALE RATS (SECOND EXPERIMENT).

The haematocrit and serum cholesterol values given are those measured at the outset, on the eighth day and on the eleventh day of the experiment respectively. All other figures refer to values measured at the conclusion of the experiment. Histograms have been prepared from these mean values. (Figure 13, facing p. 81.)

Group.	Control rats.	Hexoestrol treated rats.
No. of Rats.	4	4
Treatment.	0.1 ml. Propylene Glycol subcutaneously per day for 15 days.	850 μg./kg./ day Hexoestrol in 0.1 ml. Propylene Glycol subcutaneously for 15 days.
Body weight gain. (g.)	50	12.5
Haematocrit. (Parts per thousand.)	404 ; 440 ; 390 ;	389 ; 431 ; 380 ;
Serum cholesterol. (mg./ 100 ml.)	81.0; 66.2; 67.6;	73.2; 33.2; 23.6;
Adrenal cholesterol. (g./ 100 g. tissue.)	2.87	0.515
Liver cholesterol. (mg./ 100 g. tissue.)	234	161
Liver glycogen. (g./ 100 g. tissue.)	9.21	5.61
Relative rate of hepatic synthesis of cholesterol from acetate.	1.0	0.20

TABLE 14. THE ADMINISTRATION OF HEXOESTROL TO ADRENALECTOMIZED MALE RATS (FIRST EXPERIMENT).

Histograms have been prepared from the mean values of serum cholesterol. (Figure 14, facing p.82.)

Group.	Control.		Control-Adrenalectomized.		Adrenalectomized Hexoestrol-treated.	
Treatment.	Untreated.		Adrenalectomized. 0.1 ml. Propylene Glycol /rat /day subcutaneously for 6 days.		Adrenalectomized 540 µg./ kg./ day Hexoestrol in 0.1 ml. Propylene Glycol subcutaneously for 6 days.	
No. of Rats.	3		2		3	
Period.	Initially.	Terminally.	Initially. (while intact.)	Terminally.	Initially. (while intact.)	Terminally.
Body weight. (g.)	175	190	193	212	187	147
Serum cholesterol (mg./ 100 ml.)	55.2	60.5	66.9	42.3	50.2	6.12
Haematocrit. (Parts per thousand.)	435	480	465	450	440	465

TABLE 15. THE ADMINISTRATION OF HEXOESTROL TO ADRENALECTOMIZED MALE RATS. (SECOND EXPERIMENT.)

Histograms have been prepared from the mean values of serum cholesterol. (Figure 15, facing p.82.)

Group.	Adrenalectomized.		Adrenalectomized Hexoestral-treated.	
Treatment.	Adrenalectomized. 0.1 ml. Propylene Glycol / rat / day subcutaneously for 6 days.		Adrenalectomized. 400 µg./ kg./ day Hexoestrol in 0.1 ml. Propylene Glycol subcutaneously for 6 days.	
No. of Rats.	5		6	
Period.	Initially. (intact.)	Terminally.	Initially. (intact.)	Terminally.
Body weight (g.).	248	245	249	220
Serum cholesterol (mg./ 100 ml.)	62.9	62.7	62.6	17.7
Haematocrit. (Parts per thousand.)	489	456	483	473

TABLE 16, (Part 1). ADMINISTRATION OF NATURAL OESTROGENS TO YOUNG MALE RATS.

The oestrogens were injected subcutaneously in propylene glycol. Histograms have been prepared from the mean terminal values of serum cholesterol. (Figure 16, facing p. 83.)

Duration of experiment. (Days.)	0	6	13	21	27	0	6	13	21	27
	Serum cholesterol. (mg./ 100 ml.)					Haematocrit. (Parts per thousand.)				
Control rats. (4) 0.1 ml. propylene glycol/rat / day subcutaneously for 31 days.	73.8	74.4	67.9	74.9	58.7	444	480	455	484	471
Oestrone rats. (4) 3.3 mg./ kg./ day oestrone for 16 days, then 6.4 mg./ kg. / day for 15 days.	66.6	57.7	57.7	63.3	56.8	433	460	420	406	418
Oestradiol rats. (4) 1.9 mg./ kg./ day oestradiol for 16 days then 3.6 mg./ kg. / day for 15 days.	67.8	71.1	67.6	64.8	61.1	420	458	408	440	413
Oestriol rats. (4) 4.7 mg./ kg./ day oestriol for 16 days then 9.1 mg./ kg. / day for 15 days.	76.5	41.8	56.8	52.7	60.4	395	426	407	414	419

TABLE 16. (Part 2). ADMINISTRATION OF NATURAL OESTROGENS TO YOUNG MALE RATS.

The treatment of the groups of rats has been described in the first part of table above. Histograms have been prepared from some of these mean values, all of which were determined at the conclusion of the experiment. (Figure 16, facing p. 83.)

Group.	Body weight gain. (g.)	Adrenal cholesterol. (g./ 100 g. tissue.)	Liver cholesterol. (mg./ 100 g. tissue.)	Liver glycogen. (g./ 100g. tissue.)	Relative rate of hepatic synthesis of cholesterol from acetate.
Control rats. (2)	34	3.26	193	3.77	1.0
Oestrone rats. (2)	3	1.49	373	1.98	2.2
Oestradiol rats. (2)	8	0.959	284	2.75	0.48
Oestriol rats. (2)	11	1.20	300	2.32	0.45

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ACKNOWLEDGMENTS.

The author wishes to express his appreciation of the advice and guidance of Professor G.F.Marrian, F.R.S., and Dr. G.S.Boyd under whose supervision this investigation was carried out. Thanks are also due to Dr. J.W.Minnis for his assistance in making the radio-activity measurements and to Messrs. J.F.Syme and W.C.Cooper for valuable technical assistance.

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